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SOME OF THE FACTORS INFLUENCING THE GROWTH OF MOLDS IN BUTTER

L. C.

A DISSERTATION

Submitted to the Graduate Faculty in
Candidacy for the Degree of

DOCTOR OF PHILOSOPHY

by

HAROLD MACY

Reprinted from Technical Bulletin 64 of the
Minnesota Agricultural Experiment Station,
St. Paul, Minnesota
December, 1929

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SOME OF THE FACTORS INFLUENCING THE GROWTH OF MOLDS IN BUTTER[1]

HAROLD MACY

INTRODUCTION

The market value of butter is determined, in a large measure, by flavor and aroma, but the general appearance and physical structure also have considerable influence upon a critical and discriminating buyer.

One of the most disturbing defects of market butter is due to the growth of molds, which produce discolored areas on the surface of the butter, wrapper, or packing, often sufficiently serious to cause rejection by the dealer. On the other hand, the mold growth may be such that there are no marked, visible signs of molding to mar the appearance of the butter, but quite enough to bring about decided, deleterious changes in the flavor and aroma. The discoloration due to the growth of molds is always a serious matter, but when the flavor and aroma may be affected simultaneously, or even independently, as they are by the development of certain molds, the situation assumes very serious proportions. As a rule the market has been thinking of the molding of butter principally as a defect in appearance, because this is the most striking manifestation of the development of these microörganisms.

Generally, moldy butter occurs as an isolated case, altho at times an outbreak of moldiness from certain creameries may assume the proportions of an epidemic. When such a situation arises, losses to the creameries are often heavy, involving hundreds or thousands of dollars, quite sufficient to cause a minor financial crisis for large creameries and a major crisis for smaller establishments. In recent years, moldy butter has attracted much more attention than it did formerly. The reason for this is not entirely clear, altho changes in manufacturing methods and a more discriminating market may have had a part in this increased interest.

The appearance of mold has been more frequent in unsalted butter, and occurs most commonly during the early spring and summer months. However, isolated cases may occur at any season of the year and in various grades of butter and margarine.

The mold problem is really serious for the creamery industry, considering the damage done by the mold in producing the typical surface discoloration and deterioration of the product. A number of investi-

[1] The material in this bulletin was submitted to the Graduate Faculty of the Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy, granted June, 1929.

gators have studied some of the factors that may influence the growth of mold in butter, but some factors have not been studied carefully, and must be understood clearly before the industry controls the situation. For instance, it has been generally agreed that salt has a preservative action in butter and tends to prevent moldiness. Nevertheless, many cases of mold occur in highly salted butter. The problem is not simple, nor will it be easy to solve. So many factors may influence the growth of molds in butter that each must be studied thoroly before a proper evaluation can be made for individual species and mixtures of species that may occur in a given lot of butter. The relationship of these factors, one to the other, must be established before the problem can be approached with a reasonable degree of assurance that it can be solved and control exercised.

The studies reported here were undertaken in an attempt to provide data from which future research may be directed toward a more complete solution of the problem.

REVIEW OF LITERATURE

There is no indication in the literature as to the original observation of the molding of butter. Undoubtedly, mold growth occurred on butter made under primitive conditions and stored where there was every opportunity for growth. For many years articles on dairying have referred to the appearance of discolored areas on butter, but before mycological laboratory methods were perfected little definite information was available. Saccardo (111) in 1886 stated that *Oospora ruberrima* had been reported by Trabut as occurring on butter in Algiers. Duclaux (34) in 1886 mentioned the fact that molds were found in butter. In 1892, Siedel (124) found that granular butter, stored for several months in brine, changed in flavor and developed roquefort, turnipy, or beet flavors, which he attributed to molds. McWeeney (80) in 1894 described and illustrated a fungus, causing dark brown spots on butter exhibited at Cambridge, that he considered to be like *Dematium pullulans* DeBary, but which was identified as *Cladosporium herbarum* Link. Reference to mold in butter was made from Sweden anonymously (4) and by Wågepetersen (141). Shaw (123) reported that several samples of butter in Portland, Oregon, developed bluish-black isolated spots on the surface, which eventually became covered with the growth of a fungus identified as *Stemphylium butyri*. He also stated that a similar case had been noted in North Carolina. In an extended report upon experimental exports of unsalted butter to the London market, published by the United States Department of Agriculture, Alvord (2) stated that results were unsatisfactory because a portion of the butter became moldy. In a monograph that has been

quoted widely, Gripenberg (53) discussed the investigations carried on during the years 1896-1898 in Finland to determine the causes of moldy butter. The molding of butter had become a serious problem for the exporters of Finnish butter and demanded some study. During the last 30 years there have been increasing numbers of references to the appearance of mold in butter, and results of research upon sources, species of molds, factors influencing growth, chemical activity, and methods for control have been published. Many of these will be discussed.

FACTORS INFLUENCING THE GROWTH OF MOLDS ON BUTTER

Many factors may be considered as affecting the growth of molds, among which the food supply, moisture, atmospheric conditions, temperature, and miscellaneous physical and chemical environment are the most noteworthy. Furthermore, the species of mold undoubtedly is an important factor. These factors will be considered separately.

Food Supply

Butter carries a variety of food elements—fats, proteins, carbohydrates, and mineral salts—which should provide nutriment for the molds that may be present.

1. **Fats and related substances.**—As fat is the principal constituent of butter, it is well to consider the part which fat may play in the nutrition of some of the common fungi. In an investigation of the use of fats by certain fungi, Schmidt (117) found that when various oils were added to a mineral solution, the molds grew and utilized, especially, the neutral fats. The decomposition of a glyceride was considered as beginning with a splitting into glycerol and the fatty acids, which in turn might be attacked. If the oil covered the surface of the nutrient solution, the molds were shut off from oxygen and did not grow. Duclaux (35) melted a sample of butter and decanted a portion of the fat. The remainder of the sample was emulsified and inoculated with a species of Penicillium. Changes took place in the fats and there were variations in the proportion of fatty acids released. Butyrin was saponified more easily by the culture than caproin, but both were found to be more readily attacked than the other glycerides. Later, Duclaux (36) found that if an oil was impure and carried a substance that imbibed water, the surface of the substrate might be covered after a few days by an abundant vegetation in which *Penicillium glaucum* predominated. Water played an important rôle in this growth of mold especially as it might carry various nutrients and furnish moisture for germination. In a study of rancidity of butter, Hanus (56) reported that the glycerides of the saturated fatty acids

were decomposed by molds but that those of the unsaturated acids were not. He pointed out later (57) that the molds first attacked the lactose and proteins and later decomposed the fats. Eichholz (39) (40) made the statement that microörganisms could not grow in pure butterfat but that *Penicillium glaucum* developed especially well and caused an intense roquefort flavor in butter rich in casein. Crampton (25) (26) concluded from his experiments with moldy oleomargarine that where nitrogenous or other non-fatty material afforded a nutritive medium for the growth of molds, edible fats could be hydrolyzed so that fatty acids and glycerol were liberated. Laxa (72) found that molds brought about an important decomposition of butterfat; but that the splitting of all glycerides did not take place in the same degree. The glycerides of the insoluble fatty acids that have higher molecular weights were found to be more easily hydrolyzed by the molds than those of lower molecular weights, and the toxicity of the freed, soluble fatty acids increased with the increasing molecular magnitude. Schreiber (118) found that pure fat by itself was not a foodstuff for the microörganisms that he studied, but where other nutrients were available molds were able to split the fat. As a result of his experiments Rahn (99) concluded that the decomposition of fat took place only in the presence of organic nitrogen. Kühl (70) (71) reported that molds isolated from butter were able to attack fat, but that this process took place after the molds had made their original development on the curd of butter. Roussy (109) investigated the value of fatty substances as food for *Phycomyces nitans, Rhizopus nigricans,* and *Sterigmatocystis nigra,* and stated that for these molds fat was just as good food as carbohydrates. His studies (110) were continued with similar results when other species were used. Söhngen (125) found that several molds were capable of producing lipase, which split the fat in butter and margarine. In extensive investigations Spieckermann (126) (127) studied the effect on fats of cultures of *Penicillium glaucum* and other molds. The fats were split in a variety of ways when some nitrogenous compounds were present in the substrate. Batten and Bywaters (9) in experiments with sterile cacao butter, observed that molds would not grow on it at any temperature unless water was present. Colonies did appear on a solidified emulsion of the cacao butter and water (about 30 per cent) after 3 months at room temperature. When small quantities of sterile prune juice agar were mixed with cacao butter, vigorous growth was observed in less than a week. Bywaters (21) also stated that pure fats were very resistant to the attack of fungi, probably because of the lack of nitrogenous and other elements required for growth. As a result of researches on the rancidity of vegetable margarine, Jacobson (62) decided that the influence of molds on absolutely dry cocoanut oil was of no significance, but if only traces

of water were present rancidity might occur. Stokoe (129) found that *Penicillium glaucum* when inoculated into pure fats produced little change, but when the fats were emulsified with nutrient media the mold developed within a few days. He (130) stated that the development of rancidity in water-free fats was not due to the activity of microörganisms. The products formed as a result of the action of *Penicillium sp.* were discussed in a later publication by the same author (131). Flieg (49) studied the action of *Aspergillus niger* on a variety of fats in a synthetic medium that encouraged the early growth of the fungus, and found that the different fats were attacked in different ways. After reviewing the literature extensively, Stärkle (128) stated that the presence of water and nitrogenous substances was necessary for the growth of molds in cocoa fat. Zikes (147) likewise made the statement that pure fats and waxes did not support the growth of fungi and that positive results in the past were probably due to adherent traces of proteins and carbohydrates. In a recent publication, Patil and Hammer (92) reported that butterfat in a purified condition seemed quite resistant to the growth of microörganisms. Strained fat and ghee kept much better than butter or ghee containing added water.

Schmidt (117) found that when a mineral nutrient was provided, oleic and palmitic acids were more or less satisfactory sources of carbon for *Aspergillus niger,* after glycerides were broken down into the component acids and glycerol. According to Bokorny (14) butyric acid may be used as food for molds, but Bitting (11) found that a 0.2 per cent solution retarded the growth of Penicillium, while *Oidium lactis* did not develop in more than 0.1 per cent. Crampton (26) found that *Coniothecium sp.* attacked the fatty acids freed in oleomargarine, with a preference for those of lower molecular weights. Rahn (99) agreed that fungi show a preference for the lower acids. Laxa (72) reported the utilization of volatile fatty acids released in the splitting of butterfat by molds. Spieckermann (126) found that certain molds were able to use fatty acids, especially if they were finely divided. Roussy (110) was able to obtain good growth of several species of mold on oleic, palmitic, and stearic acids when they were used in connection with Raulin's medium. Tausson (132) reported that oleic, stearic, and palmitic acids were utilized by *A. flavus,* altho the saturated acids were more suitable than the oleic acid. Kiesel (66) was of the opinion that fatty acids show considerable toxicity toward *Aspergillus niger,* especially those containing the most carbon. In this respect the molecular structure also appeared to be significant. The differences in toxicity were explained on the basis of the differences in penetrability of the protoplasmic layer of the cell for various substances.

The glycerol released by the splitting of fats could be readily utilized by fungi, according to Burr (19) who found that *Penicillium glaucum* and *Aspergillus niger* would grow in 43 per cent glycerol solutions. Ehrlich (38) demonstrated that *Oidium lactis* utilized glycerol as a food. Roussy (110) came to the conclusion that the several species of molds which he was studying developed much better on fatty acids than on glycerol, with the exception of species of Penicillium and Aspergillus. Glycerol did not serve particularly well as a source of carbon, according to Schmidt (117).

When ivory-nut oil and lecithin were added to a nutrient medium, von Euler (44) found that the growth of *Penicillium glaucum* and *Rhizopus chinensis* was extensive after the medium was irradiated by ultra-violet light.

Apparently, pure fat is not available for direct use by molds; sufficient quantities of water and nutrient substances appear to be necessary before the splitting and utilization of the fat can be accomplished. There is some question as to the action of molds upon the free fatty acids. A difference appears to exist between the value of the different acids, because of their composition, structure, or some other factor. Large quantities might be toxic and small amounts stimulating. Apparently glycerol and lecithin are able to furnish a satisfactory food supply for many molds.

2. **Proteins and related substances.**—The literature abounds with references to experiments undertaken to determine the type of nitrogen compound most useful for various species of mold. They are so extensive that no attempt will be made to review them. As normal butter contains a considerable quantity of rich nitrogenous material in the form of the droplets of buttermilk, it may be assumed that the molds will find a variety of proteins and related compounds sufficient for growth, providing the molds are in such a position that they are able to obtain the substances directly.

The question may arise as to the possibility of molds fixing nitrogen when they are attempting to grow in such a substance as pure butterfat. Dox (32) (33) reported that he found no evidence that *Aspergillus fumigatus* could fix nitrogen. The literature upon the subject of nitrogen fixation has been reviewed very thoroly by Duggar (37) who came to the conclusion that nitrogen fixation could not be established for *Aspergillus niger*, *Penicillium digitatum*, *Penicillium expansum*, and some other forms, while *Phoma betae* showed signs of fixation when growing on mangel and sugar beet decoction with sugar.

As far as the utilization of the protein fraction of butter is concerned, Batten and Bywaters (9), Burr and Wolff (19), Crampton (26), Eichholz (40), and Laxa (73), all expressed the opinion that a high curd content favored the growth of molds in butter. Boekhout

and de Vries (12) held that the percentage of curd in itself was not a factor, because the merest traces of curd were sufficient to encourage the growth of mold. This observation seems to be more in accordance with the facts relating to the quantities of nitrogenous material necessary for the development of fungi.

3. **Carbohydrates and mineral salts.**—The value of various carbohydrates and mineral elements or salts as food for molds has been studied by many workers. It would be impractical to present a review of the voluminous literature upon this subject. In connection with studies on butter, it was reported anonymously (5) that lactose was not a good source of food for *Oidium lactis* or *Mucor mucedo*, but was fairly satisfactory for *Penicillium glaucum*. In solutions containing sugar, a marked submerged mycelial development was noted. This observation is of interest in light of the findings presented later. Boekhout and de Vries (12) reported that lactose was not the best type of sugar for *Hormodendrum cladosporioides*. In his studies on the influence of lactose on the decomposition of casein by microörganisms, Laxa (73) found that proteolysis was favored by the presence of lactose. According to Ehrlich (38) *Oidium lactis* was able to utilize lactic acid that might be formed by the hydrolysis of lactose.

The fact that for years the majority of studies on molds have been made upon synthetic media containing a variety of inorganic salts, is evidence that mineral elements are satisfactory and necessary food sources, if they are present in the proper compounds and in the proper quantities. Normal buttermilk carries a variable quantity yet more or less satisfactory variety of most of the inorganic elements essential for the development of many fungi.

As a whole, butter contains a variety of food elements, some of which are readily available for use. Much depends upon the molds being properly oriented so that they are in intimate contact with the most easily utilizable compounds. In butter, the protein, carbohydrate, and mineral salts are in the buttermilk, which is finely dispersed throughout the mass of butterfat. As pointed out by Rahn and Boysen (100) these particles may be extremely small and so numerous that the majority of the droplets of buttermilk are sterile. Further, some of the droplets may be largely water with the slightest traces of foodstuffs, if any, unless the spores of the molds have germinated and produced sufficient mycelium and enzymes to attack the abundant supply of adjacent fat.

Moisture

It is generally recognized that moisture is necessary for the germination of the spores of molds and that the development of hyphae is

accelerated when sufficient moisture is available. In order to utilize any foodstuff, water in sufficient quantities must be present. This has been indicated in connection with the availability of fat as a nutrient for fungi.

Moisture in substrate.—Batten and Bywaters (9) prepared a series of blocks of cacao butter containing from 0 to 20 per cent of water in an emulsified state and inoculated mold cultures into the center of the blocks. Under these conditions, the growth of the mold was slow, no matter how high the percentage of water. This may have been explained by other factors, such as a lack of suitable food or oxygen. Mold did develop on solidified emulsions of cacao butter and water (about 30 per cent) when spores were inoculated on the surface. Burr and Wolff (19) found that when butter contained water in large droplets instead of being in the normal, finely divided state described by Boysen (15) and Rahn and Boysen (100), mold development was favored. They pointed out that nutrients were not available unless sufficient moisture was present. Combs and Eckles (23) made the statement that moisture content is a factor governing the development of molds in butter. As pointed out elsewhere, Duclaux (36) found that water was required for the germination of mold spores. According to Gripenberg (53), wood and paper used in butter packages sustained the growth of molds when sufficient amounts of moisture were present. Hood and White (60) were of the opinion that normal butter contained sufficient moisture for mold development. This seems to be certain, as the moisture in butter is present largely in connection with the principal food consituents such as proteins, carbohydrates, and mineral matter. In his investigations on the causes of rancidity of vegetable margarine, Jacobson (62) found that oils become rancid through mold action when small quantities of water (0.2 to 0.5 per cent) were present. König, Spieckermann, and Bremer (68) reported that a multiplication of molds occurred in three sorts of cottonseed meal only when the water content was higher than 14 per cent. McWeeney (80) warned against superfluous moisture in butter if molding were to be prevented. As pointed out elsewhere, Patil and Hammer (92) observed the development of microörganisms in butterfat and ghee only when water was present with these substances. Thom and Shaw (140) reported mold in butter with a water content ranging from 7.38 to 18 per cent. According to Welte (145), *Penicillium glaucum* made good growth after 4 days on bread containing 33 per cent of water, but less satisfactory development in 6 days when the water content was 20 to 25 per cent. With *Aspergillus nidulans*, no growth was obtained, even after 6 days, when 25 per cent or less of moisture was present. He quoted Flügge as authority

for the statement that mold growth was completely hindered when the water content was as low as 10 to 12 per cent.

It is evident that water in the substrate is essential for the development of molds. Just what the minimum moisture requirement may be under different conditions is not so clear. The fact remains, however, that normal butter contains a high percentage of moisture in proportion to the most easily utilized food constituents and should provide a satisfactory substrate for mold growth from the standpoint of moisture.

Moisture in atmosphere.—The matter of humidity is another consideration of some importance. Combs and Eckles (23) held that the moisture content of the atmosphere in which butter was stored had a considerable influence upon the development of molds. This is in agreement with the observations of Orla-Jensen (63) that *Cladosporium butyri, Oidium lactis,* and *Penicillium glaucum* grew best when butter was kept in a moist room. According to König, Spieckermann, and Bremer (68), molding was increasingly abundant with an increase in humidity. Macy and Pulkrabek (81) stored samples of unsalted butter wrapped in mold-contaminated parchment under conditions of varying humidity. Nineteen samples kept at 1° C. for 50 to 60 days at approximately 70 per cent relative humidity showed no mold. The same was found true of ten samples stored at 12° C. for 30 days at about this humidity; two other samples at the same temperature and humidity after 50 to 80 days were moldy. A high relative humidity (90 to 100 per cent) favored the growth of molds upon comparable samples kept at the same temperatures. Thom and Shaw (140) found that samples of butter inoculated with various molds and kept at room temperature for several days showed no growth of mold at ordinary humidities, but when the samples were placed in a moist chamber the growth was active. At the low humidities none of the cultures appeared to develop, regardless of the moisture content of the butter. The failure of mold to grow on butters with high protein content when kept at low humidities indicated that the moisture in the atmosphere was the essential factor, as the cultures developed in the moist chamber in butters with a low protein content. Studies were also reported in which definite humidities, namely, 100, 90, 79.6, and 69.6 per cent were employed. The growth of molds was found to be greatest at 100 per cent, good at 90 per cent, considerable at 79.6 per cent, and little or none at 69.6 per cent relative humidity. Unquestionably, humidity is a very important factor in the growth of molds in butter.

Atmosphere

The importance of a sufficient supply of oxygen for the development of molds in butter has been suggested by a number of investigators, among whom are, Boekhout and de Vries (12), Burr (18), Burr and Wolff (19), Duclaux (36), Gripenberg (53), Hood and White (60) and Rogers (108).

Lopriore (78) observed that the germination of spores of *Mucor mucedo* was slowed by the presence of 10 per cent carbon dioxide in the atmosphere. Undiluted CO_2, altho producing total inhibition, did not kill the spores even after an exposure of three months. The formation of sporangia was more readily suppressed than spore germination. Brown (17) reported that within quite wide limits, the oxygen pressure had very little effect upon the germination and growth of certain molds, such as Botrytis, Fusarium, and Alternaria. The germination and growth of these organisms was retarded by CO_2, especially at lower temperatures with a scanty food supply.

According to Porodko (95), it did not appear that aerobic organisms such as *A. niger, P. glaucum,* and *M. stolonifer* were particularly sensitive to changes in oxygen pressure. Sevenster (120) (121) (122) stated that butter stored under vacuum was not in a favorable environment for the growth of molds.

Karsner and Saphir (65) studied the effect of high partial pressures of oxygen upon several species of molds grown on Sabouraud's agar in petri dishes kept in glass jars. They found that concentrations of oxygen of 76 per cent or more exercised a definite inhibitory effect upon some molds.

Rippel and Bortels (106) observed that the development of *Aspergillus niger* from spores was hampered by removing the CO_2 from the atmosphere. They held that CO_2 was necessary for the functioning of plant cells.

According to Rockwell and Highberger (107) a species of Mucor was found to be inhibited in growth when incubated at 37° C. in a jar containing 4 per cent NaOH to remove some of the CO_2 from the atmosphere.

The notion that molds are strict aerobes seems to be faulty, but it is evident that they do require some oxygen for their normal development, altho excessive quantities are inhibitory. How important these facts may be in connection with the molding of butter is not fully explained. One must bear in mind that a certain amount of air is entrapped in ordinary butter, the amount and distribution varying at different times as shown by Rahn and Mohr (102). The extent of the surface of butter exposed to the air also may be a factor. Reisz (104) has shown that a cylinder (r^2 h) of butter exposes 14.5

per cent and a cube (r^3) 24.1 per cent more surface than a sphere ($4/3\ r^3$). If air is essential for the development of mold in butter, the shape of the package may be worthy of consideration.

Temperature

Temperature profoundly influences the growth of microörganisms. Molds are no exception to this.

Welte (145) reported that *Penicillium glaucum* and *Mucor stolonifer* showed the best growth at room temperature; *Aspergillus nidulans* grew well at 36° to 37° C. In his studies on *Aspergillus niger* and *Penicillium glaucum,* Thiele (135) observed that the maximum temperature for growth was not constant and depended upon the medium. The minimum for *P. glaucum* was 1.5° to 2.0° C. and for *A. niger,* 6° to 8° C. Later, he reported (136) that *P. glaucum* made only scanty growth at 30° to 40° C. Thom (137) found that few Penicillium species grew normally at 37° C. but nearly all showed rapid growth at 12° to 30° C. The development was progressively reduced by lower temperatures. At 10° to 20° C. it was slow but good, while at temperatures approaching 0° C. the growth was very much slower. Ames (3), in studying the minimum temperature for germination of spores of *Monilia fructigena, Penicillium digitatum, Rhizopus nigricans,* and other storage-rot fungi, found that the spores of these species had a minimum germination point at 1° C., 1° C., and 3° C. while their optimum temperatures for growth were 25° C., 25° C., and 35° C., respectively. He pointed out that the minimum temperature for fructification in all cases was several degrees higher than that for growth. Brooks and Cooley (16) determined that the spores of *Alternaria sp., Botrytis cinerea,* and *Penicillium expansum* germinated slowly at 0° C. on cornmeal agar. *Aspergillus niger* failed to germinate at 10° C. Meyer (84) advanced the opinion that it was not permissible to speak of an optimum temperature for the growth of fungi except in the sense that it was the temperature which permitted the greatest rate of growth under strictly specified conditions other than temperature.

These reports are representative of many that indicate that each species shows different responses to different temperatures for germination, growth, or fructification.

In respect to the growth of molds in butter at different temperatures, the reports are largely in generalities, altho Boekhout and de Vries (12) reported that moldiness had appeared in samples of butter stored at −3° to −6° C. for 4 weeks to 4½ months. A report from the United States Department of Agriculture (6) recommended that butter be stored below 2° F. (−16.67° C.) to prevent mold development. Hood and White (60) pointed out that molds grew in butter over a wide range of temperatures but were checked at temperatures approach-

ing the freezing point of water. This was in agreement with the views of Rogers (108) expressed several years before. Macy and Pulkrabek (81) observed that temperature was a factor which influenced the development of mold on experimental butters. Stokoe (129) remarked that temperature entered into the factors favoring the production of rancidity of oleomargarine by molds, on the basis that this defect was more prevalent in the summer months.

In a general way it appears evident that low temperatures impede the development of molds in butter but experimental evidence for different species and specific temperatures is lacking. Butter has been found to be moldy even at relatively low temperatures when stored for sufficient periods of time.

Miscellaneous Chemical and Physical Environment

Many factors may be considered under this category, but only a few will be discussed.

Salt.—Investigations have been undertaken regarding the effect of salt (sodium chloride) upon the growth of molds. Eschenhagen (43) reported that *Aspergillus niger* would grow in a 17 per cent solution of sodium chloride in an inorganic medium, Penicillium at 18 per cent, and *Botrytis cinerea* at 12 per cent. Kosinski (69) was of the opinion that a 0.26 molar solution (1.523 per cent) of sodium chloride did not affect the respiration of *Aspergillus niger*. According to Gustafson (54), spores of *Aspergillus niger* failed to germinate in 0.5 molar solution of sodium chloride (about 3 per cent). Lindet (76) explained the antiseptic action of salt upon the basis of its depriving microörganisms of a portion of their elementary structure through plasmolysis. Wöltje (146) studied the effect of various concentrations of sodium chloride in different media upon the growth of eighteen species of Penicillium. The killing concentration of salt at 15° to 17° C. for conidia was found to be from 6 to 26 per cent and for mycelium from 8 to 27 per cent, depending upon the species. Extensive microscopic studies of the effect of salt upon the growth of *Penicillium expansum*, *Alternaria solani*, and *Oidium lactis* were reported by Bitting (11). She found that salt retarded growth and stunted development. It took longer for the conidia to germinate in higher salt concentrations. *Oidium lactis* was most susceptible and did not grow in 15 per cent salt. Molliard (85) found that sodium chloride at concentrations up to 10 per cent lowered the activity of *Sterigmatocytis nigra*. Golding (51) studied four species of Penicillium isolated from blue-veined cheese and inoculated into sweet skimmilk and Czapek's medium containing 4, 8, 12, and 16 per cent of salt. The salt decreased the power to digest casein as well as the growth of these organisms.

Observations have been made in a general way upon the effect of salt on the appearance of mold in butter. Alvord (2) reported that unsalted butter exported to England from the United States became moldy. Dean and Harcourt (29) found that salt was more effective in preventing mold than other preservatives. Burr and Wolff (19) (20) reported that salted butter with a normal curd content was not an exceptionally good substrate for molds and that mold spores could not germinate well when 2 per cent salt was present in the butter. Species of Mucor did not develop in salted butter and the growth of Penicillium was retarded. Rahn, Brown, and Smith (101) found that *Oidium lactis* increased in unsalted but not in salted butter. The keeping quality of butter and margarine was improved when they contained about 3 per cent of salt according to Fischer and Gruenert (48). Hastings (59) held the opinion that mold spores could not germinate in salted butter. Combs and Eckles (23) also concluded that the salt content of butter had a considerable influence upon the development of mold. Jacobson (62) reported that vegetable margarine was well preserved only when salt or other preservative was dissolved in the water droplets so that the molds were restrained. Abbott and Ashenfelter (1) made the observation that mold counts increased more rapidly in unsalted than in salted butter. The idea that heavy salting of liners, wrappers, tubs, etc., as well as the soaking of parchment paper in brine decreased the possibilities of the development of mold upon the surface of butter or package has been upheld by anonymous writers (4), (6), (7) and by Böggild (13), Burr (18), Davis (27), Dean (28), Ibsen (61), Rogers (108), and Zoffman (148).

More detailed studies of salt effects, with special reference to the butter industry, have been undertaken. As reported elsewhere, Siedel (124) attributed to the action of molds, the roquefort, turnipy, or beet flavor that developed in granular butter immersed in 10 per cent and saturated brine. While he did not record actual visible growth, he implied that some development had occurred. Gripenberg (53) made use of a butter "serum," which consisted of the portion of butter removable after butter was melted and most of the fat decanted. When this serum contained 18 and 20 per cent of salt, molds (*Penicillium sp.* and *Trichosporium sp.*) were found growing in it after 5 to 6 months. When this material was diluted, the fungi grew much better. Hanging drops of butter serum containing 0, 10, and 25 per cent of salt were inoculated with *Penicillium crustaceum* and *Trichosporium collae*. In the unsalted serum, growth was active after 9 days for both types; with 10 per cent salt the growth was slight; with 25 per cent no signs of growth were evident. Parchment paper dipped in salted butter serum showed no molding in the 24 per cent serum but increasingly heavy growth with decreasing amounts of salt. McKay

and Larsen (79) studied the growth of *Penicillium glaucum* in media containing various quantities of salt. The growth after 2 days was luxuriant with less than 9 per cent salt present, noticeable with 9 per cent, but only a trace with 10 per cent salt. Fettick (45) followed the development of *Oidium lactis* and *Penicillium glaucum* in unsalted butter and in butter containing 3 per cent salt by plating samples at the beginning of the storage period and after 7 weeks, 2 months, and 4 months. In the salted butter the molds decreased immediately and disappeared within the 2 months; in the unsalted sample, they increased steadily. He also prepared sterile butter containing 0, 0.5, 1, 2, 3, 4, 5, and 6 per cent of salt. These samples were inoculated with *Oidium lactis, Mucor mucedo,* and *Penicillium glaucum* and stored for one week in a dark place at 17° C. Platings were made at the end of this period. All three species decreased in proportion to the increased percentage of salt. No growth appeared in samples containing 4 per cent or more of salt. The effect of various percentages of salt in Czapek's medium was studied by Thom (138). Petri plate cultures were prepared of twenty-one species of Penicillium and ten of Aspergillus, using Czapek's solution with and without agar, and containing 10 per cent of sodium chloride. After 19 days all the Penicillium and Aspergillus cultures had grown on the solid medium, but only sixteen of the Penicillium and nine of the Aspergillus cultures in the liquid. The extent of growth varied with the different species. Growth of *P. pinophilum, P. lilacinum, P. luteum, P. digitatum, P. purpurogenum, P. roseum, P. duclauxii, A. nidulans, A. fumigatus,* and a check culture of *O. lactis* was stopped or reduced to a negligible amount in the liquid medium. Further, twelve species of Penicillium were inoculated on Czapek's medium containing 0, 5, 10, and 15 per cent of sodium chloride. After 34 days, growth had occurred in all samples reported. There was relatively less development, however, with increasing percentages of salt. It was determined that *Oidium lactis* was reduced to negligible growth when the amount of salt exceeded 6 per cent.

Thom and Shaw (140) investigated the growth of three cultures of Alternaria, four of Mucor, two of *P. roqueforti,* and individual cultures of the following, *Cunninghamiella sp., Fusarium sp., O. lactis, Penicillium sp., P. expansum, P. stoloniferum, P. chrysogenum, P. purpurogenum, Rhizopus nigricans, Trichoderma sp.,* and a red mold on Czapek's agar containing 6.5 per cent and 14.4 per cent of salt. The plates were kept in a moist chamber. All grew in 6.5 per cent of salt, Oidium least and Penicillium most. In 14.4 per cent of salt, Alternaria and Penicillium alone developed, the latter showing the better growth. In an 18 per cent salt Czapek's agar, three species of Penicillium and *Aspergillus repens* grew; in 21 per cent the spores of *P.*

chrysogenum were the only ones to germinate. Thom and Shaw noted that the growth was better on salted agar than on salted butter of the same percentage of salt. Denning (30) found butter in good condition after 2 months' storage at 50° F. in a 20 per cent solution of sodium chloride. Later experiments (31) indicated that butter kept in 30 per cent of salt had been preserved in good condition. *Hormodendrum dadoporioides* cultures were seeded by Boekhout and de Vries (12) into a medium containing peptone, levulose, and nutrient salts and varying quantities of sodium chloride. Five cultures grew in the medium containing 13.4 per cent salt, four in 14.3 per cent, two in 16 per cent, two in 17 per cent, and none in 18, 19, or 20 per cent. Samples of butter containing 0, 2, 2.5 and 3 per cent of salt were placed in Erlenmeyer flasks at 21° C. and held for one month after being inoculated with two Hormodendrum cultures. Growth occurred only on the unsalted butter. It is evident that Boekhout and de Vries obtained better growth on the salted, synthetic medium.

Paraschchuk (1901) (91) studied the growth of several species of mold on malt agar containing varying percentages of salt and found that *Penicillium glaucum* and *Cladosporium herbarum nigr.* were able to develop when the salt concentration was as high as 18 per cent.

According to the foregoing evidence, the influence of salt varies, however, depending upon the species and the substrate. Altho unsalted butter is most commonly affected, mold often appears on salted butter. This occurrence demands explanation.

Degree of acidity.— Schaffer (115) stated that the growth of molds was rather favored by the low acidity of butter made from pasteurized cream. According to Laxa (73) the presence of lactic acid did not hinder markedly the peptonization of casein by molds. In studies carried on by Boekhout and de Vries (12) two cultures of *Hormodendrum dadoporioides* were grown on a base medium containing lactic acid. One culture was checked by 0.75 per cent lactic acid; the other not until the percentage of acid reached 1.0 per cent.

None of these investigations were based upon carefully controlled conditions in butter, so give no satisfactory idea of the effect of acidity in butter upon the growth of molds.

Many papers dealing with the effect of hydrogen-ion concentration upon the development of molds have been presented in recent years. Only two are mentioned here. Gustafson (55) studied the effect of various hydrogen-ion concentrations upon the respiration of *Penicillium chrysogenum*. He found that the rate of respiration was not affected within the range of pH 4.0 and pH 8.0. Johnson (64) at the Iowa station, reviewed previous investigations and reported that the acid and alkaline reactions inhibiting the growth of seven molds ranged from pH 1.6 to pH 11.2 depending upon the species.

These investigations indicate that most molds grow over a wide range of hydrogen-ion concentrations. Most species are favored by an acid medium, and the limits of acidity reached in ordinary butter are such that the hydrogen-ion concentration, as such, should not have much influence on mold development. The nature of the substances bringing about the changes in pH appears to be of much greater significance.

Light.—Altho the general opinion, expressed by Rogers (108), that molds developed much better in the dark than in the light, has been held by most investigators, Kolkwitz (67) and Maxinow (82) suggested that at times light accelerated the metabolism of fungi. In practice, however, butter is kept in packages and in storage rooms where light does not gain direct access to it. Consequently, light would not be expected to influence the growth of molds on butter.

Vitamins.—There have been some investigations into the effect of growth-stimulating substances, such as vitamins, upon the development of molds. Linossier (77) pointed out that *Oidium lactis*, *Aspergillus niger*, and *Penicillium glaucum* were able to grow in pure culture in media lacking vitamins but containing necessary nutrients. When the nutrients were greatly reduced, the addition of vitamin-containing substances, such as orange juice, sometimes stimulated growth. Lepeschkin (75) reviewed the literature on the subject and concluded that vitamins were not of great importance in the nutrition of molds.

At any rate, butter ordinarily contains vitamins and in sufficient quantities and variety to satisfy the slight demands of the fungi.

Species of Molds Isolated from Butter

Many species of molds have been isolated from butter but they have not always been shown to be the specific causes of defects in appearance, flavor, or aroma. The species of molds are important, as one must determine what types actually grow in butter even under the most favorable conditions. Many forms encountered when butter is plated may exist in butter only in the form of spores, which do not germinate under the conditions existing in the product. Every species should be studied to determine whether or not it can develop in ordinary butter.

It is not necessary to present anything more than a catalog of some of the species that have been isolated from butter, giving the species name applied by the investigator who reported it and the key number to locate the authority in the bibliography. In addition, attention may be called to the fact that See (119) described several species isolated from paper. It is possible that some of these might be found on parchment paper used for wrapping butter.

Alternaria sp. (22) (140); *Aspergillus sp.* (18) (26) (90) (91) (128) (129); *Aspergillus flavus* (9); *Aspergillus glaucus* (113) (129); *Aspergillus niger* (19) (62); *Aspergillus oryzae* (9); *Botricanus scostan* (90) (91); *Botrytis sp.* (58); *Chaetomium sp.* (36); *Cladosporium sp.* (90) (91) (129) (140); *Cladosporium butyri* (62) (63); *Cladosporium herbarum* (80) (90) (91); *Coniosporium sp.* (26); *Coniothecium sp.* (26); *Dematium sp.* (70); *Dematium pullulans* (80); *Epicoccum sp.* (22); *Eurotium repens* (68); *Eurotium rubrum* (68; *Hormodendrum cladosporioides* (12); *Monilia sp.* (83) (90) (91) (96); *Monilia alba* (90) (91); *Monilia candida* (90) (91); *Monilia roseum* (90) (91); *Mucor sp.* (18) (22) (53) (56) (58) (90) (91) (105) (140); *Mucor hiemalis* (90) (91); *Mucor mucedo* (5) (19) (46) (57) (133); *Mucor petrinsularis* (36); *Mucor racemosus* (90) (91); *Mucor spinosus* (36); *Mucor sylvaticus* (90) (91); *Oidium lactis* (5) (8) (22) (39) (45) (58) (63) (83) (86) (87) (88) (89) (90) (91) (96) (101) (105) (113) (133) (140); *Oidium variicolor* (97); *Oospora ruberrima* (111); *Penicillium sp.* (18) (19) (26) (35) (53) (58) (90) (91) (105) (128) (131); *Penicillium brevicaule* (142) (144); *Penicillium chrysogenum* (139); *Penicillium crustaceum* (53) *Penicillium expansum* (140); *Penicillium glaucum* (5) (9) (19) (36) (39) (40) (45) (46) (62) (63) (70) (90) (91) (113) (129) (130) (133); *Penicillium olivaceum* (?) (80); *Penicillium roqueforti* (22) (70) (140); *Rhizopus arirus* (90) (91); *Stemphylium butyri* (93) (123) (140); *Sterigmatocystis sp.* (36); *Stilbaciae graphium* (90) (91); *Trichosporium collae* (53); *Verticillium sp.* (36).

EXPERIMENTAL

Purpose of Study

The purpose of the studies reported on subsequent pages was to determine the influence of certain factors upon the growth of molds with special reference to their development in butter; and to investigate some of the basic considerations with a hope that the results might offer opportunities for their interpretation in terms of the complex inter-relationships of the various factors that appear to be operating in such a valuable and concentrated food as butter. It was anticipated that many phenomena would appear to throw light on some of the perplexing problems at present recognized and to serve as bases upon which further studies might rest.

Method of Procedure

In the beginning, it was necessary to decide what factors appeared to be most important in their influence upon the growth of molds, particularly in butter. These were as follows: (1) Food supply, (2) mois-

ture, (3) temperature, (4) atmosphere, and (5) miscellaneous chemical and physical factors, especially the effect of various concentrations of sodium chloride.

Species of Molds Used in Experiments

In order to have one fixed condition throughout the experiments, ten species of molds were selected to be used in the studies. These cultures were representative of types of molds found commonly in butter. As a convenience in the presentation of the experimental data, reference will be made to them by number. These cultures and their corresponding key numbers are as follows:

1. *Alternaria humicola* Oudemans
2. *Aspergillus flavus* Link
3. *Aspergillus niger* Van Tieghem
4. *Hormodendrum cladosporioides* (Fresenius) Saccardo
5. *Mucor sylvaticus* Hagem
6. *Oospora lactis var. A.* (Fresenius) Lindau
7. *Oospora lactis var. B.* (Fresenius) Lindau
8. *Penicillium biforme* Thom
9. *Penicillium expansum* Link
10. *Rhizopus nigricans* Ehrenberg

The Mucor and Rhizopus stock cultures were carried on potato-dextrose agar, the Oospora cultures on whey agar, and the rest on Czapek's agar. These media gave the most luxuriant growth for the particular species. Inoculations in the various experiments were made directly from cultures 10 days to 2 weeks in age, particular care being taken to transfer none of the culture medium.

Methods Employed in Experiments

The methods employed for preparing the different substrata, for maintaining the desired temperature, humidity, etc., will be discussed under the respective subdivisions of the material presented.

Manner of Recording Results

The extent of growth made by the ten species on different substrata and under other varying conditions will be recorded uniformly by the following symbols:

−	No visible growth
±	Questionable growth
+	Slight, visible growth
++	Moderate growth
+++	Abundant growth
++++	Normal growth

Note: Wherever these symbols are in parentheses, they indicate subsurface growth.

In a similar manner, the color produced by the cultures under the various conditions will be registered according to the following symbols:

W —White	B —Black
Y —Yellow	Br—Brown
G —Green	C —Cream

EXPERIMENTAL RESULTS

The experimental data will be presented under the following subdivisions:

A. Food supply
   1. Fats and related substances
   2. Proteins and related substances
   3. Carbohydrates and related substances
   4. Mineral constituents
   5. Combinations of 1, 2, 3, and 4

B. Moisture
   1. Moisture in substrate
   2. Moisture in atmosphere

C. Temperature

D. Atmosphere
   1. Ordinary air supply
   2. Reduced air supply—partial vacuum
   3. Partial removal of carbon dioxide
   4. Removal of oxygen

E. Miscellaneous chemical and physical factors
   1. Salt content

A. Food Supply

The primary requirement for the growth of microörganisms is a satisfactory food supply. Butter furnishes a variety of foodstuffs, principally fat, but also proteins, carbohydrates, and mineral salts in varying amounts. Each of these constituents has been studied as a source of food for the ten species of molds previously selected.

1. Fats and Related Substances

a. Fresh Butterfat

Methods. Fresh unsalted butter was melted at a temperature of 50° to 55° C. and placed in a warm, separatory funnel. The curd and water that settled out were drawn off. The fat was washed five times in the funnel by shaking with it equal quantities of water at 60° to 65° C. After each washing, the mixture was allowed to stand until the layers of fat and water were distinct, whereupon the water was drawn from the funnel. The washed fat was tempered to 55° C. and left at this temperature for 24 hours, during which time it was filtered

through filter paper to remove traces of water. The filtered fat was tubed in 10-cc. amounts and autoclaved. When ready to use, the fat was melted and poured into sterile petri plates and solidified quickly. Analyses of the autoclaved fat showed it to be water-free. The cultures were streaked across the surface of the fat as uniformly as possible. The plates were placed in piles on a rack in a humidor consisting of a 10-gallon, covered earthenware crock in the bottom of which was a one-inch layer of water containing sufficient bichloride of mercury to maintain it as a sterile fluid. The relative humidity, determined by a wet-dry bulb hydrometer, was maintained at 100 per cent. The temperature of incubation ranged from 20° to 25° C.

TABLE I

GROWTH OF MOLD CULTURES ON FRESH BUTTERFAT

Culture	Extent of growth at 20–25° C., high humidity, after						
	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	6 weeks
	Visible						Microscopic
1. *Alternaria humicola*	—	—	—	—	—	—	—
2. *Aspergillus flavus*	—	—	—	—	—	—	—
3. *Aspergillus niger*	—	—	—	—	—	+B	+
4. *Hormodendrum cladosporioides*	—	—	—	—	—	—	+
5. *Mucor sylvaticus*	—	+	+	+	+	+	+
6. *Oospora lactis var. A.* ..	—	—	—	—	—	—	—
7. *Oospora lactis var. B.* ..	—	—	—	—	—	—	+
8. *Penicillium biforme*	—	—	—	—	—	—	+
9. *Penicillium expansum*	—	—	—	—	—	—	+
10. *Rhizopus nigricans*	—	—	—	—	—	—	—
Check	—	—	—	—	—	—	—

Results. The extent of growth in the fresh butterfat is indicated in Table I. After one week the only visible growth was noted with Culture 5 and it was evident that development was restricted to the point of inoculation, where sufficient residual nutriment may have been present in the mycelium inoculated. At the end of 6 weeks, Culture 3 had produced a few black sporangia; Culture 5 had shown no further increase, rather a slight diminution in the mass of growth. A microscopic examination of the plates after 6 weeks incubation revealed signs of germination of the conidia in Cultures 3, 4, 5, 7, 8, and 9, but very little hyphal development except in Culture 5, where the mycelium had developed extensively.

b. Old Butterfat

Methods. The method for preparing the old butterfat was the same as that described for fresh butterfat. The butter used was unsalted, one month old, and decidedly cheesy in flavor. The surface was discolored with spots of mold. The conditions for inoculation and incubation were the same as those for the fresh butterfat.

Results. As shown in Table II, growth was evident after 4 days in Cultures 3 and 8, but again this development was at the point of inoculation. Culture 3 produced a few black sporangia; Culture 8 formed a light green spot. At the end of one week, Culture 10 showed slight aerial mycelium, which disappeared within the following week. Culture 1 began to produce a green spot after 3 weeks. This became deeper in color during the subsequent period. Culture 9 developed a small green spot after the third week. A dark green streak appeared along the line of inoculation in Culture 4 at the expiration of the 6 weeks. The microscopic examination indicated that Cultures 1, 2, 3, 4, 8, and 9 had made slight growth during the trial. In general, the growth on this type of butterfat was somewhat better than that on the fresh butterfat.

TABLE II

GROWTH OF MOLD CULTURES ON OLD BUTTERFAT

Culture*	Extent of growth at 20–25° C., high humidity, after						
	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	6 weeks
	Visible						Microscopic
1. *Alt. humicola*	—	—	—	+G	+	+	+
2. *A. flavus*	—	—	—	—	—	—	+
3. *A. niger*	+B	+	+	+	+	+	+
4. *H. cladosporioides*	—	—	—	—	—	+G	+
5. *M. sylvaticus*	—	—	—	—	—	—	—
6. *O. lactis var. A.*	—	—	—	—	—	—	—
7. *O. lactis var. B.*	—	—	—	—	—	—	-
8. *P. biforme*	+G	+	+	+	+	+	+
9. *P. expansum*	—	—	—	—	+G	+	+
10. *R. nigricans*	—	+W	—	—	—	—	—
Check	—	—	—	—	—	—	—

* Abbreviations used in this and subsequent tables are as follows: Alt.=Alternaria, A.=Aspergillus, H.=Hormodendrum, M.=Mucor, O.=Oospora, P.=Penicillium, R.=Rhizopus.

c. Butterfat from Washed Cream

Methods. Four pounds of 30 per cent sweet cream were diluted to 3 gallons with ordinary tap water and heated to 35° C. The diluted cream was then separated in a centrifugal separator. The resultant cream was again diluted in the same way, heated, and separated. The process was repeated until the cream had been diluted and separated ten times. The cream that was finally obtained was placed at 12° C. and allowed to stand over night. During this period the fat had formed a solid mass, floating above a slightly cloudy serum. The mixture was heated to 50° C. and placed in a warm separatory funnel. In this way it was possible to remove the serum. The fat was washed five times with water at 60° to 65° C. Thereafter, it was placed at 55° C. for 24 hours, during which time it was allowed to filter through ordinary filter paper. An analysis showed that all water had been removed. The fat was placed in test tubes and autoclaved. When desired for use the fat was melted, poured into petri plates, and solidified promptly. Con-

ditions of inoculation and incubation were the same as those described in the preceding experiments.

Results. As in the previous experiment, Cultures 3 and 8 gave evidence of growth after 4 days, according to the data presented in Table III. Culture 10 had developed a noticeable aerial mycelium during this period but, as happened before, this mycelium gradually disappeared. A scanty aerial mycelium was sent up by Culture 5 after one week, but this likewise disappeared after 4 weeks. A green streak following the line of inoculation of Culture 4 appeared at the end of 4 weeks, while Culture 9 had produced a slight green spot during the same period. The microscopic examination made after 6 weeks showed that Cultures 2, 3, 4, 5, 8, 9, and 10 had been able to develop slightly near the point of inoculation.

TABLE III

GROWTH OF MOLD CULTURES ON BUTTERFAT FROM WASHED CREAM

Culture	Extent of growth at 20–25° C., high humidity, after						
	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	6 weeks
	Visible						Microscopic
1. *Alt. humicola*	−	−	−	−	−	−	−
2. *A. flavus*	−	−	−	−	−	−	+
3. *A. niger*	+B	+	+	+	+	+	+
4. *H. cladosporioides*	−	−	±	±	+G	+	+
5. *M. sylvaticus*	−	+W	+	+	+	−	+
6. *O. lactis var. A.*	−	−	−	−	−	−	−
7. *O. lactis var. B.*	−	−	−	−	−	−	−
8. *P. biforme*	+G	+	+	+	+	+	+
9. *P. expansum*	−	−	−	±	+G	+	+
10. *R. nigricans*	+W	+	−	−	−	−	+
Check	−	−	−	−	−	−	−

d. Butterfat Washed with Alcohol

Methods. A portion of the fresh butterfat used in the experiment reported in Table I was mixed with equal parts of 95 per cent ethyl alcohol and shaken thoroly. The fat was drawn off by means of a separatory funnel, placed at 55° C. and filtered through paper, after which it was placed on a water bath to drive off any remaining alcohol. The final analysis showed no traces of water in the fat. The fat was tubed and autoclaved. When desired, it was melted and poured into petri dishes, where it solidified. The fat was inoculated as described in preceding experiments.

Results. Table IV gives the results of the experiment. Culture 3, as before, produced a few black sporangia after 4 days. At the end of one week, Culture 5 had sent forth scanty aerial mycelium but, as had happened in the previous experiments, this disappeared after 4 weeks. Culture 2 appeared to grow on this preparation of butterfat and after one week a few yellowish-green sporangia were visible. After 3 weeks, Culture 4 developed to such an extent that green streaks were evident,

and Cultures 8 and 9 produced green spots. Finally, at the end of 6 weeks, Culture 1 produced a small green spot. The microscope revealed extensive development of Culture 4 and slight mycelial growth of Cultures 1, 2, 3, 8, and 9. In general, the growth was slightly better in the fat washed with alcohol than on the other preparations.

TABLE IV

GROWTH OF MOLD CULTURES ON BUTTERFAT THAT HAD BEEN WASHED WITH ALCOHOL

Culture	Extent of growth at 20–25° C., high humidity, after						
	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	6 weeks
	Visible						Microscopic
1. *Alt. humicola*	—	—	—	—	—	+G	+
2. *A. flavus*	—	+G	+	+	+	+	+
3. *A. niger*	+B	+	+	+	+	+	+
4. *H. cladosporioides*	—	—	—	+G	+	+	++
5. *M. sylvaticus*	—	+W	+	+	+	—	—
6. *O. lactis var. A.*	—	—	—	—	—	—	—
7. *O. lactis var. B.*	—	—	—	—	—	—	—
8. *P. biforme*	—	—	—	—	+G	+	+
9. *P. expansum*	—	—	—	—	+G	+	+
10. *R. nigricans*	—	—	—	—	—	—	—
Check	—	—	—	—	—	—	—

e. Fresh Butterfat Plus Water

Methods. The fresh butterfat, as prepared in experiment "a" described previously, was used in the trial. In order to provide a substrate in which the fat and water might be more intimately associated and in a more finely divided state, a medium containing 1.5 per cent of washed agar was prepared. Fifteen grams of Bacto agar were placed in a cloth bag and suspended in running water over night in order to remove some of the soluble and finely divided impurities. After the washed agar had been dried at 55° C. it was weighed and sufficient distilled water added to make a 1.5 per cent concentration of agar. This became a substrate, which supplied sufficient water but little nutriment. Similar batches of the same medium were prepared at various times, some containing 1.0 per cent and some 1.5 per cent of agar, depending upon circumstances. This medium was placed in test tubes in measured amounts and autoclaved. When ready for use in combination with the fat, the agar and fat were melted and the butterfat was added to the agar in the proportions of four parts of agar to one part of fat. The mixture was shaken thoroly until the fat was in a finely divided state, then poured into petri dishes and cooled on cold surface as quickly as possible. The droplets of fat were evenly dispersed throughout the solidified agar. The fat-agar mixtures were inoculated by streaking the cultures across the surface. The plates were stored at room temperature (20° to 25° C.) for 4 weeks at a relative humidity varying from 60 to 65 per cent.

Results. The fact that molds made very meager growth on the washed agar substrate is indicated in Table V. The results reported

are representative of other lots of the same medium used in later experiments. Very slight growth was obtained at any time, and in most cases it was barely visible, especially on the surface, altho a slight penetration of the mycelium below the surface was noted in some instances. As shown in Table VI, the presence of water led to much more extensive growth of all the cultures except 6 and 7. Cultures 1, 3, and 4 grew especially well. It is significant that Cultures 8 and 9 were able to produce a more or less typical odor of Roquefort cheese before any growth was visible.

TABLE V

Growth of Mold Cultures on 1.5 Per Cent Washed Agar Substrate

Culture	Extent of growth at 20–25° C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks
			Visible			Microscopic
1. *Alt. humicola*	+W	+	+	+	+	+
2. *A. flavus*	+	+	+	+	+	+
3. *A. niger*	+	+	+	+	+	+
4. *H. cladosporioides* ..	+	+	+	+	+	+
5. *M. sylvaticus*	+W	+	+	+	+	+
6. *O. lactis var. A.* ...	—	—	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—	—	—
8. *P. biforme*	±	+	+	+	+	+
9. *P. expan'um*	±	±	±	±	±	+
10. *R. nigricans*	+W	+	+	+	+	+
Check	—	—	—	—	—	—

TABLE VI

Growth of Mold Cultures on a Mixture of 1.5 Per Cent Washed Agar and Fresh Butterfat

(4 parts of agar medium: 1 part of butterfat)

Culture	Extent of growth at 20–25° C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks
			Visible			Microscopic
1. *Alt. humicola*	+W	+	++G	++	++	+
2. *A. flavus*	—	+Y	+YG	+	+	+
3. *A. niger*	+B	++	++	++	++	+
4. *H. cladosporioides* ..	±	++G	++	++	++	+
5. *M. sylvaticus*	+W	+	+	+	+	+
6. *O. lactis var. A.* ...	—	—	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—	—	—
8. *P. biforme*	—*	+G	+	+	+	+
9. *P. expansum*	—*	+G	+	+	+	+
10. *R. nigricans*	+W	+	+	+	+	+
Check	—	—	—	—	—	—

* Roquefort odor.

f. Old Butterfat Plus Water

Methods. The procedure was the same as that just described under "e" except that the old butterfat prepared for experiment "b" was employed.

Results. The growth of the molds on old butterfat was increased when water was present, as the data in Table VII indicate. The de-

velopment of Cultures 1, 3, and 4 was particularly good. Cultures 6 and 7 failed to grow. It may be noted that the Roquefort cheese odor, which appeared in the fresh butterfat with water in the case of Cultures 8 and 9, was not evident in the old butterfat-water mixture.

TABLE VII

GROWTH OF MOLD CULTURES ON A MIXTURE OF 1.5 PER CENT WASHED AGAR AND OLD BUTTERFAT

(4 parts of agar medium: 1 part of butterfat)

Culture	Extent of growth at 20-25° C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks
			Visible			Microscopic
1. *Alt. humicola*	+W	+	++G	++	++	+
2. *A. flavus*	—	—	+YG	+	+	+
3. *A. niger*	+B	++	++	++	++	+
4. *H. cladosporioides* ..	±	++G	++	++	++	+
5. *M. sylvaticus*	+W	+	+	+	+	+
6. *O. lactis var. A.* ...	—	—	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—	—	—
8. *P. biforme*	—	—	+G	+	+	+
9. *P. expansum*	—	—	+G	+	+	+
10. *R. nigricans*	+W	+	+	+	+	+
Check	—	—	—	—	—	—

g. Alcohol-Extracted Butterfat Plus Water

Methods. The method of preparing the substrate was the same as that described in "e" except that the butterfat washed with alcohol (see experiment "d") was used. The methods of inoculation and incubation were the same as those followed in experiment "e."

Results. The growth of Cultures 1, 2, 3, and 4 was quite extensive, as shown in Table VIII. As before, Cultures 6 and 7 showed no signs of development. The Roquefort cheese odor was apparent in Culture 8 at the end of 4 days. In general, the growth was practically the same as when water was added to the fresh and old butterfats.

TABLE VIII

GROWTH OF MOLD CULTURES ON A MIXTURE OF 1.5 PER CENT WASHED AGAR AND ALCOHOL-WASHED BUTTERFAT

(4 parts of agar medium: 1 part of butterfat)

Culture	Extent of growth at 20-25° C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks
			Visible			Microscopic
1. *Alt. humicola*	+W	+	++G	++	++	+
2. *A. flavus*	+Y	++YG	++	++	++	+
3. *A. niger*	+B	++	++	++	++	+
4. *H. cladosporioides* ..	±	++G	++	++	++	+
5. *M. sylvaticus*	+W	+	+	+	+	+
6. *O. lactis var. A.* ...	—	—	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—	—	
8. *P. biforme*	—*	+G	+	+	+	+
9. *P. expansum*	—	+G	+	+	+	+
10. *R. nigricans*	+W	+	+	+	+	+
Check	—	—	—	—	—	—

* Roquefort odor.

h. One-half Per Cent Aqueous Emulsion of Milk Lecithin

Methods. A highly purified lecithin obtained from milk by Dr. W. E. Petersen and Dr. L. M. Thurston, of the Minnesota Agricultural Experiment Station, was emulsified by thoro shaking in water. It produced a stable, milky emulsion, which was tubed and autoclaved. After sterilization, a flocculent precipitate was formed but it remained uniformly suspended after cooling. The emulsion was inoculated from the various cultures and left at room temperature (20° to 25° C.) for 3 weeks, at a relative humidity of 60 to 65 per cent.

TABLE IX

Growth of Mold Cultures in 1.5 Per Cent Aqueous Solution of Milk Lecithin

Culture	Extent of growth at 20–25° C., ordinary humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	—	—	+W(++)*	++(++)
2. *A. flavus*	—	—	+W(+)	++YG(+)
3. *A. niger*	+WB	+	+(+)	+B(+)
4. *H. cladosporioides* ..	—	—	+G(+)	+(+)
5. *M. sylvaticus*		-	-(+)	-(+)
6. *O. lactis var. A.* ...	—	—	-(+)	-(+)
7. *O. lactis var. B.* ...	—	—	-(+)	-(+)
8. *P. biforme*	—	++G	++(+)	++(+)
9. *P. expansum*	—	—	+W(+)	+(+)
10. *R. nigricans*	—	—	-(+)	-(+)
Check	—	—	—	—

* Signs in parentheses indicate subsurface growth.

Results. Table IX gives the results obtained with this emulsion of lecithin. With Culture 3, a white mycelium and a few black sporangia appeared on the walls of the tube just above the surface of the liquid after 3 days. Culture 8 produced a good, green surface growth at the end of one week. Cultures 1, 2, and 9 produced white rings near the surface of the solution after 2 weeks, but at the end of 3 weeks were growing reasonably well on the surface. All cultures showed subsurface mycelial development, which was especially good in Culture 10. Unquestionably lecithin was a fairly good source of food for these species.

i. One-half Per Cent Aqueous Emulsion of Milk Lecithin in 1.5 Per Cent Washed Agar

Methods. A portion of the sterile lecithin emulsion, prepared according to the methods described in the preceding trial, was added to an equal amount of melted, sterile, 1.5 per cent washed agar made up in 5-cc. amounts in test tubes. After thoro mixing, the medium was slanted. The agar slants were inoculated over the surface in the usual manner. The tubes were incubated at room temperature (20° to 25° C.) for 3 weeks at a relative humidity of 60 to 65 per cent.

Results. It will be noted in Table X that growth began promptly in all cases, and became practically normal after 3 weeks with the ex-

ception of Cultures 6 and 7, which already had been shown to do very poorly on fat or fat-like substrata. The development of the cultures was much better on the solid medium than on the fluid preparation.

TABLE X

GROWTH OF MOLD CULTURES ON MIXTURE OF 1.0 PER CENT WASHED AGAR AND 0.5 PER CENT AQUEOUS SOLUTION OF MILK LECITHIN

(Equal parts of agar medium and lecithin solution)

Culture	Extent of growth at 20–25° C., ordinary humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	++W	+++G	+++	+++
2. *A. flavus*	+W	++Y	++YG	++
3. *A. niger*	+W	+W	+B	++
4. *H. cladosporioides* ..	+W	++G	+++G	+++
5. *M. sylvaticus*	++W	+++	+++WB	+++
6. *O. lactis var. A.* ...	+W	+	+	+
7. *O. lactis var. B.* ...	+W	+	+	+
8. *P. biforme*	+W	+	+G	++
9. *P. expansum*	+W	+	+++G	+++
10. *R. nigricans*	++W	+++	+++WB	+++
Check	—	—	—	—

j. One Per Cent Glycerol in 1.5 Per Cent Washed Agar

Methods. Chemically pure glycerol was added to melted, sterile, 1.5 per cent washed agar in amount sufficient to make a one per cent solution. This mixture was tubed, autoclaved, and slanted. The usual agar stroke was made with each culture. The cultures were then placed in a humidor, which consisted of a covered 10-gallon metal churn, equipped with a false, perforated bottom, under which was placed a quantity of water to which bichloride of mercury had been added to maintain sterility. The relative humidity was maintained at 100 per cent. The temperature of incubation was 20° to 25° C. and the storage period 3 weeks.

TABLE XI

GROWTH OF MOLD CULTURES ON 1.5 PER CENT WASHED AGAR CONTAINING 1 PER CENT GLYCEROL

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	±	+W(++)	+(+++G)	+(+++)
2. *A. flavus*	+W(+)	+G(++)	+(++)	++(+++)
3. *A. niger*	±	+B(+)	+(+)	+(++)
4. *H. cladosporioides* ..	±(+G)	+G(++)	+(+++)	+(+++)
5. *M. sylvaticus*	±(++)	+W(++)	+(++)	+(++)
6. *O. lactis var. A.* ...	+W(++)	+(++)	+(+++)	+(+++)
7. *O. lactis var. B.* ...	+W(++)	+(++)	+(+++)	+(+++)
8. *P. biforme*	±	+G(+)	+(+)	+(+)
9. *P. expansum*	±(+)	+G(++)	+(+++)	+(+++)
10. *R. nigricans*	±W(+)	+(+)	+(+)	+WB(+)
Check	—	—	—	—

Results. Table XI gives the results of this experiment. It will be observed that Cultures 2, 6, 7, and 10 showed some growth after 3 days. With the rest, growth became noticeable after one week. How-

ever, none of the cultures exhibited any further surface growth except Culture 2, which had developed extensively after 3 weeks. The significant result was the extensive subsurface growth of nearly all cultures. This subsurface development in most cases was much more marked than the surface growth. Cultures 1 and 4 produced remarkable dark green subsurface mycelium. The same phenomenon appeared in aqueous solutions of glycerol, studied in preliminary unreported trials.

k. One Per Cent Butyric Acid in 1.5 Per Cent Washed Agar

Methods. Purified butyric acid was added to measured amounts of tubed, melted, sterile, 1.5 per cent washed agar medium in quantities sufficient to make a one per cent solution. These tubes were shaken thoroly and slanted. After inoculation the cultures were placed in a metal humidor (described previously) at 20° to 25° C. for 3 weeks, and at a relative humidity of 100 per cent.

TABLE XII

GROWTH OF MOLD CULTURES ON 1.5 PER CENT WASHED AGAR CONTAINING 1 PER CENT BUTYRIC ACID

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	—	—	—	—
2. *A. flavus*	—	—	—	—
3. *A. niger*	—	—	—	—
4. *H. cladosporioides* ..	—	—	—	—
5. *M. sylvaticus*	—	—	—	—
6. *O. lactis var. A.* ...	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—
8. *P. biforme*	—	—	—	—
9. *P. expansum*	—	—	—	—
10. *R. nigricans*	—	—	—	—
Check	—	—	—	—

Results. Table XII reveals the fact that the cultures under investigation were unable to develop in this concentration of butyric acid. This is in agreement with the results of preliminary studies.

l. One Per Cent Palmitic Acid in 1.5 Per Cent Washed Agar

Methods. Purified palmitic acid was added in a sufficient quantity to a sterile, melted, 1.5 per cent washed agar medium to make a one per cent concentration. After warming the mixture in a steam bath until the palmitic acid melted, it was shaken thoroly and placed as quickly as possible in sterile tubes. The tubes were rotated thoroly so that the acid was fairly well dispersed and the medium solidified as quickly as possible in a slanted position. The acid hardened on the surface in areas ranging from 0.1 mm. to 5.0 mm. in diameter, with equal areas of clear agar between them, while the bulk of the medium was clear. The inoculations were made over the surface in such a way that the cultures covered representative areas of the solidified acid.

The tubes were incubated under the conditions prevailing in the previous experiment.

Results. The growth on the medium containing palmitic acid as the source of nutriment was fairly good, as shown in Table XIII. All cultures exhibited some surface growth, which was much better than that obtained on the agar checks. The remarkable feature of this series, however, was the extensive development of Cultures 1, 2, 4, 5, 6, and 10 below the surface. The mycelium grew directly away from the surface of the slants and in Cultures 1 and 4, the hyphae were dark green.

TABLE XIII

GROWTH OF MOLD CULTURES ON 1.5 PER CENT WASHED AGAR CONTAINING 1 PER CENT PALMITIC ACID

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	+W(++G)	+G(++)	+(++)	+(++)
2. *A. flavus*	+W(++)	+YG(++)	+(++)	+(++)
3. *A. niger*	+W(+)	+B(+)	+(+)	+(+)
4. *H. cladosporioides* ..	+W(+)	+G(++G)	+(++)	+(++)
5. *M. sylvaticus*	+W(++)	+(++)	++(++)	++(++)
6. *O. lactis var. A.* ...	−(+)	+W(+)	+(++)	+(++)
7. *O. lactis var. B.* ...	−(+)	+(+)	+(+)	+(+)
8. *P. biforme*	−	+G(+)	+(+)	+(+)
9. *P. expansum*	−	+G(+)	+(+)	+(+)
10. *R. nigricans*	+W(++)	++(++)	++WB(++)	++(++)
Check	−	−	−	−

m. One Per Cent Stearic Acid in 1.5 Per Cent Washed Agar

Methods. The one per cent concentration of stearic acid in 1.5 per cent washed agar substrate was prepared in the same manner as the preceding mixture of palmitic acid and agar. The emulsion obtained in this case, however, was more uniform. The medium became very cloudy. Inoculation and incubation conditions were identical with the foregoing "k" and "l."

TABLE XIV

GROWTH OF MOLD CULTURES ON 1.5 PER CENT WASHED AGAR CONTAINING 1 PER CENT STEARIC ACID

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	+W	+W	+WG	+G
2. *A. flavus*	−	−	-	−
3. *A. niger*	−	−	-	-
4. *H. cladosporioides* ..	−	−	−	−
5. *M. sylvaticus*	−	−	−	−
6. *O. lactis var. A.* ...	−		−	
7. *O. lactis var. B.* ...	−	−	-	−
8. *P. biforme*	−	−	−	−
9. *P. expansum*	−	-	-	−
10. *R. nigricans*	−	−	−	−
Check	−	−	−	−

Results. Table XIV gives the results of this experiment. The only surface development observed in this medium occurred with Culture 1, where a small area of growth appeared about the point of inoculation. The medium was so opaque that it was impossible to determine whether any subsurface growth had taken place.

n. One Per Cent Oleic Acid in 1.5 Per Cent Washed Agar

Methods. Purified oleic acid was measured into definite amounts of sterile, melted, 1.5 per cent washed agar medium in tubes so that the concentration of acid was one per cent. The mixture was shaken thoroly and solidified as quickly as possible in a slanting position. The oleic acid appeared in the surface in clear droplets, approximately one mm. in diameter and close together. Conditions of inoculation and incubation were exactly the same as in the three preceding trials.

TABLE XV

Growth of Mold Cultures on 1.5 Per Cent Washed Agar Containing 1 Per Cent Oleic Acid

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	+W(++)	++WG(++G)	++(++)	++(++)
2. *A. flavus*	+G(++)	++(+++)	++(+++)	++(+++)
3. *A. niger*	−(+)	+B(++)	+(++)	+(+++)
4. *H. cladosporioides* ..	+G(++)	++B(++G)	++(+++)	++(+++)
5. *M. sylvaticus*	+W(++)	++(++)	++(++)	++(++)
6. *O. lactis var. A.* ...	−(+)	+W(++)	+(++)	+(++)
7. *O. lactis var. B.* ...	−(+)	+W(++)	+(+++)	+(+++)
8. *P. biforme*	−(+)	+G(++)	+(++)	+(++)
9. *P. expansum*	−(+)	+G(++)	++(+++)	++(+++)
10. *R. nigricans*	+W(++)	++(++)	++WB(++)	++(++)
Check	−	−	−	−

Results. As indicated in Table XV the growth on this substrate was better than that obtained on media containing any of the other acids commonly found in butterfat. Surface development was good in all except Cultures 3, 6, 7, and 8. The subsurface mycelium was remarkably heavy, especially in Cultures 2, 3, 4, 7, and 9. Cultures 1 and 4 produced their characteristic dark green hyphae in the depth of the medium.

2. Proteins and Related Substances

a. One Per Cent Aqueous Solution of Peptone

Methods. A one per cent solution of Bacto peptone was prepared in distilled water, tubed, and autoclaved. The peptone solution was inoculated in the usual manner with each of the cultures. The tubes were incubated under conditions identical with those employed in the preceding experiment.

Results. The growth of the cultures began promptly in this solution as indicated in Table XVI. At the end of one week, the de-

velopment of all cultures was good, and in some cases perfectly normal. While some subsurface development was noted, it was not nearly so extensive as that on the surface.

TABLE XVI

GROWTH OF MOLD CULTURES ON 1 PER CENT AQUEOUS SOLUTION OF PEPTONE (BACTO)

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	+W(++)	++WG(+)	++WG	++G
2. *A. flavus*	++W	++WY	+++YBr	++++Br
3. *A. niger*	++W(+)	+++B(+)	++++(+)	++++(+)
4. *H. cladosporioides* ..	++G(+)	+++(+)	++++(+)	++++(+)
5. *M. sylvaticus*	++W(++)	+++(+)	+++(+)	++++(+)
6. *O. lactis var. A.* ...	+++W(++)	++++(++)	++++(++)	++++(++)
7. *O. lactis var. B.* ...	++W(++)	+++(++)	++++(++)	+++(++)
8. *P. biforme*	++G(+)	+++(+)	++++(+)	++++(+)
9. *P. expansum*	++W(+)	++++	++++	++++
10. *R. nigricans*	+++W(++)	++++(+)	++++(+)	++++WB(+)
Check	—	—	—	—

b. "Curd" Portion of Butter

Methods. Fresh, unsalted butter was melted at 60° to 65° C. and placed in a warm separatory funnel from which the so-called "curd" was withdrawn with ease, as it settled below the melted fat. This curd, which consisted principally of protein, was tubed and autoclaved. The water content was 86.3 per cent. The curd was inoculated with the test cultures. The tubes were placed in a humidor (as described under the preparation of fresh butterfat), where the relative humidity remained at 100 per cent during the storage period. The temperature of incubation was 20° to 25° C.

TABLE XVII

GROWTH OF MOLD CULTURES ON CURD PORTION OF BUTTER

Culture	Extent of growth at 20–25° C., high humidity, after			
	2 days	4 days	1 week	2 weeks
1. *Alt. humicola*	—	++++G	++++	++++
2. *A. flavus*	++W	+++YG	++++	++++
3. *A. niger*	+B	+++	++++	++++
4. *H. cladosporioides* ..	++G	++++	++++	++++
5. *M. sylvaticus*	+++WB	++++	++++	++++
6. *O. lactis var. A.* ...	++W	++	+++	++++
7. *O lactis var. B.* ...	+W	+++C	++++	++++
8. *P. biforme*	—	++++G	++++	++++
9. *P. expansum*	+G	++++	++++	++++
10. *R. nigricans*	+++WB	++++	++++	++++
Check	—	—	—	

Results. The growth of all cultures, except Cultures 1 and 8, became visible within 4 days, as is shown in Table XVII. At the end of one week, development was practically normal. The growth reached a maximum after 2 weeks and further incubation resulted in

no noticeable changes. It will be noted that growth was both rapid and luxuriant.

c. Dialyzed Curd from Butter

Methods. A portion of the curd obtained by the method outlined in the previous experiment was placed in a collodion sac and dialyzed in distilled water for 24 hours. It was then tubed and autoclaved. The final preparation contained 89.3 per cent water. The conditions of inoculation and incubation were identical with those employed in the previous trial.

TABLE XVIII

GROWTH OF MOLD CULTURES ON DIALYZED CURD FROM BUTTER

Culture	Extent of growth at 20–25° C., high humidity, after			
	2 days	4 days	1 week	2 weeks
1. *Alt. humicola*	—	+++G	++++	++++
2. *A. flavus*	+W	+++YG	+++	+++
3. *A. niger*	+W	++++B	++++	++++
4. *H. cladosporioides* ..	+++G	++++	++++	++++
5. *M. sylvaticus*	++W	++++WB	++++	++++
6. *O. lactis var. A.* ...	+W	++	+++	+++
7. *O. lactis var. B.* ...	+W	++	+++	+++
8. *P. biforme*	—	+++G	++++	++++
9. *P. expansum*	—	+++G	+++	+++
10. *R. nigricans*	+W	+++WB	++++	++++
Check	—	—	—	—

Results. The growth of the molds did not begin as promptly as it did on normal curd preparations, as indicated in Table XVIII. After 4 days, however, the growth was marked and after one week it reached a maximum. In general, the development was not quite so vigorous as that observed on untreated curd.

d. Washings from Cream

Methods. The sera (obtained from the successive washings of cream as explained in a previous experiment) were collected after each separation, tubed, and autoclaved. The first, third, sixth, and tenth rinsings were selected for this purpose. The first washing appeared quite milky, resembling diluted skimmilk. The tenth washing was practically clear. The washings were inoculated in the usual manner with the various cultures. The tubes were kept at 20° to 25° C. for one week, at a relative humidity of 60 to 65 per cent.

Results. Table XIX gives the results obtained when the washings were inoculated with the test cultures. Growth was practically normal in the serum from the first washing after one week, with the exception of Cultures 6 and 7. In the third washings, Cultures 1, 8, and 9 were the only ones to show any development and this was rather scanty. Culture 1 was able to grow on the sixth and tenth washings; none of the others produced any visible growth with the exception of a slight

development of Culture 8 on the tenth washing. It was apparent that most of the nutrients must have been removed from the cream by the early washings.

TABLE XIX

GROWTH OF MOLD CULTURES ON WASHINGS FROM CREAM

Culture	Extent of growth at 20–25° C., ordinary humidity							
	1st washing		3rd washing*		6th washing*		10th washing*	
	3 days	1 week	3 days	1 week	3 days	1 week	3 days	1 week
1. *Alt. humicola*	+++G	++++	+	++	+	+	+	+
2. *A. flavus*	++Y	++++	—	—	—	—	—	—
3. *A. niger*	—	++B	—	—	—	—	—	—
4. *H. cladosporioides*	++++G	++++	—	—	—	—	—	—
5. *M. sylvaticus*	+++W	+++	—	—	—	—	—	—
6. *O. lactis var. A.*	—	+W	—	—	—	—	—	—
7. *O. lactis var. B.*	+W	+	—	—	—	—	—	—
8. *P. biforme*	+++G	++++	+G	+	—	—	—	+G
9. *P. expansum*	+G	++++	+G	+	—	—	—	—
10. *R. nigricans*	++W	++++	—	—	—	—	—	—
Check	—	—	—	—	—	—	—	—

* All cultures on 3rd, 6th, and 10th washings showed slight subsurface growth.

3. Carbohydrates and Related Substances

a. One Per Cent Aqueous Solution of Lactose

Methods. A one per cent aqueous solution of chemically pure lactose was prepared in distilled water, tubed, and autoclaved. The tubes were inoculated in the usual manner and kept for 3 weeks at a temperature of 20° to 25° C. and a relative humidity of 100 per cent, in the metal humidor previously described.

TABLE XX

GROWTH OF MOLD CULTURES ON 1 PER CENT AQUEOUS SOLUTION OF LACTOSE

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	—(+)	+W(+)	++G(++G)	++(++)
2. *A. flavus*	—(+)	+W(+)	+(+)	+(+)
3. *A. niger*	—(+)	+W(+)	+(+)	+(+)
4. *H. cladosporioides*	—	—	+G	+(+)
5. *M. sylvaticus*	—(+)	+W(+)	+(+)	+(+)
6. *O. lactis var. A.*	—(+)	—(+)	—(+)	+W(+)
7. *O. lactis var. B.*	—	—(+)	—(+)	+W(+)
8. *P. biforme*	—(+)	+W(+)	+(+)	++WG(++)
9. *P. expansum*	—(+)	+W(+)	+(+)	+(+)
10. *R. nigricans*	—(+)	+W(+)	+(++)	+(++)
Check	—	—	—	—

Results. Table XX indicates that the only signs of growth after 3 days incubation took place in the depth of the liquid or downward from the surface, where the conidia floated. At the end of one week, feeble surface growth was noted except with Cultures 6 and 7. The subsurface development was somewhat more extensive. After 3 weeks, Cultures 1 and 8 had sent up considerable mycelium from the surface,

but at the same time the subsurface growth was equally good. In general, the development in the lactose solution was not marked at any time.

b. One Per Cent Aqueous Solution of Lactose in 1.5 Per Cent Washed Agar

Methods. One gram of chemically pure lactose was added to 100 cc. of 1.5 per cent washed agar, and the mixture was tubed, autoclaved, and slanted. The usual inoculations were made and the tubes incubated under conditions described in the previous trial.

TABLE XXI

GROWTH OF MOLD CULTURES ON 1.5 PER CENT WASHED AGAR CONTAINING 1 PER CENT LACTOSE

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	−(+)	+W(++)	+(+++G)	+(+++)
2. *A. flavus*	+W(+)	+YG(++)	+(++)	+(+++)
3. *A. niger*	+W(+)	+B(+)	+(++)	+(++)
4. *H. cladosporioides* ..	+G(++G)	+(++)	+(+++)	+(+++)
5. *M. sylvaticus*	+W(++)	+(++)	+(++)	+(++)
6. *O. lactis var. A.* ...	+W(++)	+(++)	+(++)	+(++)
7. *O. lactis var. B.* ...	+W(++)	+(++)	+(++)	+(++)
8. *P. biforme*	+G(++)	+(++)	+(+++)	+(+++)
9. *P. expansum*	+G(+)	+(++)	+(+++)	+(+++)
10. *R. nigricans*	+W(+)	+(+)	+(+)	+(+)
Check	−	−	−	−

Results. The surface growth was slight, even after 3 weeks incubation, as shown in Table XXI. Subsurface development in most cases was marked. Cultures 1 and 4 produced dark green mycelium that penetrated deep into the solid medium, at right angles with the side walls of the tube. This is illustrated plainly in Plates I and II. The growth resembled that obtained on solid media containing glycerol.

c. One Per Cent Lactic Acid in 1.5 Per Cent Washed Agar

Methods. Purified lactic acid was added to measured quantities of sterile, melted, 1.5 per cent washed agar in tubes, in amounts sufficient

TABLE XXII

GROWTH OF MOLD CULTURES ON 1.5 PER CENT WASHED AGAR CONTAINING 1 PER CENT LACTIC ACID

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	−	−	+G	+G
2. *A. flavus*	+W(+)	+(+)	+Y(+)	+YG(+)
3. *A. niger*	+W(+)	+B(++)	++(++)	++(++)
4. *H. cladosporioides* ..	−	+G	++(+)	++(+G)
5. *M. sylvaticus*	−	−	−	−
6. *O. lactis var. A*	−	−	−	−
7. *O. lactis var. B.* ...	−	−	−	−
8. *P. biforme*	+G(+)	+(++)	+(++)	+(++)
9. *P. expansum*	−	+W(+)	+G(+)	+(+)
10. *R. nigricans*	−	+W	+	+
Check	−	−	−	−

to make a one per cent solution. After these sugars the tubes were cleared. The slants were inoculated in the usual manner and incubated at 20° to 25° C. for 3 weeks in a metal humidor described elsewhere, in which the relative humidity was maintained at 100 per cent.

Results. The results of this trial are given in Table XXII. It will be noted that Cultures 3 and 4 made moderate, Cultures 1, 2, 8, 9, and 10 slight, and Cultures 5, 6, and 7 no growth on the medium. The sporulation development was particularly good in Cultures 3 and 8. Cultures 4 and dark green hyphae into the depth of the medium. Where growth appeared it was better than that obtained on the pure agar substrate.

d. Diffusate from Curd of Butter

Methods. The diffusate obtained on the dialysis of the curd from butter, as explained in previous pages, was tubed and sterilized. The diffusate contained 3.37 per cent of water and 0.0238 per cent of nitrogen at the time of inoculation. It was incubated and kept at 20° to 25° C. for 3 weeks in a humidor at a relative humidity of 100 per cent.

TABLE XXII

Growth of Mold Cultures on Diffusate from Curd of Butter

Culture	Extent of growth at 20°-25° C., high humidity, after				
	2 days	4 days	1 week	2 weeks	3 weeks
1. *Alt. herbarum*	−−W	+−−	+−−	+−−	+−−−G
2. *A. flavus*	−−Y	+−YG	+−	+−	+−
3. *A. niger*	−B	−	−	−	−
4. *H. cladosporioides*	−−G	+−	+−	+−	+−
5. *M. spinosus*	−W	−	−	−	−
6. *O. lactis* var. *A.*	−W	−	−	−	−
7. *O. lactis* var. *B.*	−	−	−	−W	−
8. *P. biforme*	−−G	+−	+−	+−	+−
9. *P. expansum*	−G	+−	+−	+−	+−
10. *P. nigricans*	−W	−	−	−	+−−
Check	−	−	−	−	−

Results. Table XXIII gives the results of the experiment. It will be noted that growth began promptly except with Culture 7, which did not produce visible mycelium until the second week. Cultures 2, 3, 5, 6, 8, and 9 reached the point of maximum development after 2 days. The growth of Cultures 1, 4, and 10 became especially luxuriant. Apparently the food materials were readily available, as evidenced by the prompt growth, but were as rapidly utilized by certain of the cultures that development reached an early maximum.

4. Mineral Constituents

a. Milk Ash in 0.75 Per Cent Aqueous Solution

Methods. One part of fresh skimmilk was evaporated to dryness over a water bath, the residue incinerated in a muffle furnace and the

ash recovered. A 0.75 per cent aqueous solution was prepared, tubed, and autoclaved. The inoculated tubes were incubated at 20° to 25° C. for 2 weeks, at a relative humidity of 60 to 65 per cent. At the end of 2 weeks, one cc. of sterile skimmilk was added to each tube to determine whether the cultures were in a viable condition.

TABLE XXIV

Growth of Mold Cultures on 0.75 Per Cent Aqueous Solution of Milk Ash

Culture	Extent of growth at 20–25° C., ordinary humidity, after			Growth 3 days after adding 1 cc. sterile skimmilk
	4 days	1 week	2 weeks	
1. *Alt. humicola*	—	—	—	+++G
2. *A. flavus*	+	+	+	++YG
3. *A. niger*	—	—	—	+B
4. *H. cladosporioides* ..	—	—	—	+++B
5. *M. sylvaticus*	—	—	—	++W
6. *O. lactis var. A.* ...	—	—	—	++W
7. *O. lactis var. B.* ...	—	—	—	+W
8. *P. biforme*	—	—	—	+++G
9. *P. expansum*	—	—	—	+++G
10. *R. nigricans*	—(+)	—(+)	—(+)	+++W
Check	—	—	—	—

Results. As shown in Table XXIV, no growth occurred with most of the cultures. There was a slight subsurface mycelium in Culture 10, and a barely visible development in Culture 2. Three days after the sterile skimmilk was added, excellent growth was obtained from all except Cultures 2 and 7.

b. Milk Ash in 0.75 Per Cent Aqueous Solution Neutralized with Hydrochloric Acid

Methods. The solution of milk ash, prepared according to the method outlined in the foregoing experiment, was neutralized with hydrochloric acid. The methods of tubing, sterilizing, and inoculating, and the conditions of incubation were identical with those followed in the previous trial.

TABLE XXV

Growth of Mold Cultures on 0.75 Per Cent Aqueous Solution of Milk Ash Made Neutral with Hydrochloric Acid

Culture	Extent of growth at 20–25° C., ordinary humidity, after			Growth 3 days after adding 1 cc. sterile skimmilk
	4 days	1 week	2 weeks	
1. *Alt. humicola*	++W	++	++	+++WG
2. *A. flavus*	+W	+	+	++WY
3. *A. niger*	+WB	+	+	++B
4. *H. cladosporioides* ..	—	+W	+	++G
5. *M. sylvaticus*	—	—(+)	—(+)	++W
6. *O. lactis var. A.* ...	—	—	—	+++W
7. *O. lactis var. B.* ..	—	—	—	+W
8. *P. biforme*	++W	++	++	+++G
9. *P. expansum*	++W	++	++	+++G
10. *R. nigricans*	—(+)	—(+)	—(+)	+++W
Check	—	—	—	—

Results. It will be noted from Table XXV that fairly good growth took place after 4 days in Cultures 1, 8, and 9, while Cultures 2 and 3 showed scanty mycelial development. At the end of 2 weeks all cultures except 6 and 7 exhibited some degree of growth. Cultures 5 and 10, however, produced only subsurface mycelium. The addition of sterile milk showed that none of the cultures had been killed; all grew well in 3 days, with Culture 7 again the least active.

5. Combinations of Various Foodstuffs

a. Emulsion of Butterfat, Milk Lecithin, and Water

Methods. Five parts of old, sterile butterfat, prepared according to the methods described on page 23, were mixed thoroly with one part of a one per cent sterile aqueous emulsion of lecithin, and the mixture was poured into petri plates. These plates were inoculated by streaking the cultures across the surface, and were incubated at 20° to 25° C. for 3 weeks at a relative humidity of 100 per cent in the earthenware humidor previously described.

TABLE XXVI

GROWTH OF MOLD CULTURES ON EMULSION OF BUTTERFAT AND ONE PER CENT AQUEOUS SOLUTION OF MILK LECITHIN

(5 parts of butterfat: 1 part of lecithin solution)

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	+W	+	+	++
2. *A. flavus*	—	—	—	—
3. *A. niger*	—	—	—	+B
4. *H. cladosporioides* ..	—	—	—	+G
5. *M. sylvaticus*	—	—	—	—
6. *O. lactis var. A.* ...	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—
8. *P. biforme*	—	—	—	—
9. *P. expansum*	—	—	—	—
10. *R. nigricans*	—	—	—	—
Check	—	—	—	—

Results. The growth on the mixture of butterfat, lecithin, and water was not much better than that obtained on fat alone, as is clearly demonstrated in Table XXVI. Culture 1 began its development promptly and showed the best growth. Cultures 3 and 4 produced a scanty but visible surface growth.

b. Butterfat Plus Dry Milk Ash

Methods. Some of the old butterfat used in previous experiments was melted, poured into petri plates, and solidified promptly. Dry milk ash was sprinkled freely over the surface. The cultures were streaked across the surface in such a way that they came into contact both with the ash and the fat. The plates were incubated for 3 weeks in the

earthenware humidor at a temperature of 20° to 25° C. and a relative humidity of 100 per cent.

Results. Table XXVII gives the results obtained. The growth was somewhat better than that on pure butterfat. Visible mycelium was observed in Cultures 1, 2, 3, 4, 8, and 9 after 3 weeks incubation. Cultures 5, 6, and 7 showed no signs of development when the plates were examined under the microscope; Culture 10 gave evidence of the germination of some of the conidia and abortive mycelium.

TABLE XXVII

GROWTH OF MOLD CULTURES ON BUTTERFAT WITH MILK ASH SPRINKLED OVER SURFACE

Culture	Extent of growth at 20–25° C., high humidity, after			
	1 week	2 weeks	3 weeks	3 weeks
		Visible		Microscopic
1. *Alt. humicola*	+W	+G	++G	+
2. *A. flavus*	—	+YG	+	+
3. *A. niger*	++B	++	++	+
4. *H. cladosporioides* ..	—	—	++G	+
5. *M. sylvaticus*	—	—	—	—
6. *O. lactis var. A.* ...	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—
8. *P. biforme*	—	—	+G	+
9. *P. expansum*	—	—	+G	+
10. *R. nigricans*	—	—	—	+
Check	—	—	—	—

c. Mixture of Butterfat, Milk Ash, and Water

Methods. Two parts of sterile fresh butterfat and one part of milk ash (prepared in accordance with methods described on page 39) were mixed thoroly and added to four parts of a sterile, melted, 1.5 per cent washed agar medium. The mixture was shaken thoroly and poured into petri plates. After it had become hard, the cultures were streaked across the surface. The plates were incubated for 4 weeks at a temperature of 20° to 25° C. and a relative humidity of 60 to 65 per cent.

TABLE XXVIII

GROWTH OF MOLD CULTURES ON 1.5 PER CENT WASHED AGAR WITH FRESH BUTTERFAT AND SATURATED SOLUTION OF MILK ASH ADDED

(4 parts of agar medium: 2 parts of butterfat and 1 part of ash solution)

Culture	Extent of growth at 20–25° C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks
			Visible			Microscopic
1. *Alt. humicola*	+W	+G	+	++	++	+
2. *A. flavus*	+Y	++	++YG	++	++	+
3. *A. niger*	+B	++	++	++	++	+
4. *H. cladosporioides* ..	+G	++	++	++	++	+
5. *M. sylvaticus*	+W	+	+	+	+	+
6. *O. lactis var. A.* ...	—	—	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—	—	—
8. *P. biforme*	+W	+	+G	+	+	+
9. *P. expansum*	+W	+G	+	+	+	+
10. *R. nigricans*	+W	+	+	+	+	+
Check	—	—	—	—	—	—

Results. Table XXVIII indicates that growth began within a few days and increased slightly during the 4 weeks, with the exception of Cultures 6 and 7. Cultures 1, 2, 3, and 4 made the most noticeable progress in this medium. The microscopic examination failed to show any signs of germination of the conidia from Cultures 6 or 7. The failure of these cultures to grow on fats, minerals, or combinations of the two substances has been a regular phenomenon.

d. Aqueous Solution of Lactose and Peptone

Methods. A solution containing one per cent Bacto peptone and one per cent chemically pure lactose was prepared in distilled water, tubed, and autoclaved. After inoculation the samples were incubated at 20° to 25° C. for 3 weeks in the metal humidor at a humidity of 100 per cent.

TABLE XXIX

GROWTH OF MOLD CULTURES IN AQUEOUS SOLUTION CONTAINING 1 PER CENT LACTOSE AND 1 PER CENT PEPTONE

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola* ...	+W(+)	+++G(++)	+++(++)	++++(++)
2. *A. flavus*	++W(+)	++	+++YBr	++++
3. *A. niger*	++WB(+)	+++B(+)	++++B(+)	++++(+)
4. *H. cladosporioides*	++G(+)	+++++G(+)	++++(+)	++++(+)
5. *M. sylvaticus* ...	++W(++)	+++(++)	+++(+)	+++(+)
6. *O. lactis var. A.*	++W(++)	++++(++)	++++(++)	++++(++++)
7. *O. lactis var. B.*	++W(++)	+++(++)	+++(++)	++++(++)
8. *P. biforme*	++W(+)	++++G(+)	++++(+)	++++(++)
9. *P. expansum* ...	++W(+)	++++(+)	++++(+)	++++(+)
10. *R. nigricans*	+++W(++)	++++WB(++)	++++(+)	++++(+)
Check	—	--	—	—

Results. Table XXIX shows that all the cultures grew rapidly in the medium and soon reached a normal state of development as in peptone alone. The only effect that the lactose seemed to exert was an increase in the amount of subsurface mycelium. The protein appeared to be the more readily utilized substance.

e. Mixture of Lactose and Peptone in 1.5 Per Cent Washed Agar

Methods. Sufficient lactose and peptone were added to a 1.5 per cent washed agar medium to give a one per cent concentration of each. This mixture was tubed, autoclaved, and slanted. The slants were inoculated on the surface in the usual manner. The tubes were incubated under conditions described in the previous experiment.

Results. It will be observed in Table XXX that excellent growth was obtained in this medium. In contrast with the lactose-washed agar medium, the surface growth was predominant, altho some subsurface mycelium was produced. The contrast between the growth on the

lactose-peptone-washed agar and the lactose-washed agar is shown clearly in Plates I and III. When lactose was the main source of food, the mycelium of the molds studied seemed preferably to penetrate into the depth of the medium, but when a nitrogen compound, such as peptone, was available at the same time, the growth became characteristically concentrated on the surface.

TABLE XXX

GROWTH OF MOLD CULTURES ON 1.5 PER CENT WASHED AGAR CONTAINING 1 PER CENT LACTOSE AND 1 PER CENT PEPTONE

Culture	Extent of growth at 20–25° C., high humidity, after			
	3 days	1 week	2 weeks	3 weeks
1. *Alt. humicola* ...	+W(+)	++WG(+)	+++(+)	++++G(+)
2. *A. flavus*	++W(+)	+++WGY(+)	+++YG(+)	++++(++)
3. *A. niger*	++B(+)	++++(+)	++++(+)	++++(+)
4. *H. cladosporioides*	+++G(+Br)	++++(+)	++++(+)	++++(+)
5. *M. sylvaticus* ...	+++W(+)	+++(+)	+++(++)	+++(+++)
6. *O. lactis var. A.*	++W(+)	++(+)	++(++)	+++(++)
7. *O. lactis var. B.*	+++W(+)	+++(+)	+++(++)	+++(++)
8. *P. biforme*	++++G(+)	++++(+)	++++(+)	++++(++)
9. *P. expansum* ...	+++W(+)	++++(+)	++++G(++)	++++(++)
10. *R. nigricans*	+++W(+)	+++(++)	+++(++)	+++(++)
Check	—	—	—	—

f. Sterile, Unsalted Butter

Methods. A sample of fresh 30 per cent cream was autoclaved and churned in a sterile Dazey churn, the butter was washed with sterile water and worked with sterile paddles. Small pieces of this butter were transferred to sterile test tubes and allowed to harden over night in the cooler. After inoculation by streaking the cultures along the surface, the tubes were placed in a humidor at a temperature of 20° to 25° C. and a relative humidity of 100 per cent for 3 weeks.

TABLE XXXI

GROWTH OF MOLD CULTURES ON STERILE, UNSALTED BUTTER

Culture	Extent of growth at 20–25° C., high humidity, after			
	4 days	1 week	2 weeks	3 weeks
1. *Alt. humicola*	—	+G	+++	++++
2. *A. flavus*	—	—	+YG	+
3. *A. niger*	+B	++	++	+++
4. *H. cladosporioides* ..	+G	+++	+++	+++
5. *M. sylvaticus*	++W	+++WB	+++	+++
6. *O. lactis var. A.* ...	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—
8. *P. biforme*	—	++G	++	+++
9. *P. expansum*	+G	+++	+++	+++
10. *R. nigricans*	—	+W	+++WB	+++
Check	—	—	—	—

Results. The growth of the cultures on this combination of all of the foodstuffs previously studied is described in Table XXXI and illustrated in Plate III. The most extensive development was noted in Cultures 1, 3, 4, 5, 8, 9, and 10. The butter was discolored to the

greatest extent with Cultures 1 and 4. Culture 2 showed slight visible mycelium and a few yellowish-green sporangia. No visible growth of Cultures 6 and 7 was observed. The unsalted butter appeared to furnish ample food for the growth of the majority of the cultures studied.

B. Moisture

The influence of moisture on the growth of molds may be considered from two standpoints, namely, the moisture in the substrate itself and that carried by the atmosphere.

1. Moisture in Substrate

It has been shown in experiments reported in the previous pages that the presence of water facilitates the growth of molds on butterfat. As butter contains a considerable percentage of water, especially in intimate association with the most useful foodstuffs contained in the butter in the form of droplets of buttermilk, it was considered unessential to investigate the influence of water of constitution upon the growth of molds, further than that reported in previous or subsequent pages in connection with other factors.

2. Moisture in Atmosphere

a. Effect of Humidity on Growth of Molds on Unsalted Butter

Methods. Sweet cream, testing 35 per cent fat, was autoclaved, cooled, and churned in a sterile Dazey churn. The butter was washed with sterile water and worked with sterile paddles, due precautions being taken to prevent contamination. Small blocks of the finished butter were placed in sterile petri plates and held over night at 0° C. in order to harden the butter. The following day the cultures were streaked over the surfaces of the samples. The plates were kept at 10° to 12° C. for 6 weeks, one set in the metal humidor at a relative humidity of 100 per cent, and the other set in the laboratory at a relative humidity of 70 to 75 per cent.

Results. The comparison between the growth of the molds on the samples of butter kept at different humidities is shown in Table XXXII. At the end of 6 weeks, none of the butters kept at the lower humidity gave any evidence of growth. In the samples kept in an atmosphere saturated with moisture, Cultures 1 and 4 produced dark green smudgy areas that spread some distance from the line of inoculation; Culture 5 showed delicate, white, aerial threads of mycelium, bearing smoky-gray and black sporangia; Culture 6 produced a slight, white feltlike mass; Cultures 8 and 9 formed white and green spots scattered over the surface and also gave to the butter a distinctly Roquefort cheese

TABLE XXXII

EFFECT OF HUMIDITY UPON GROWTH OF MOLD CULTURES INOCULATED ON SURFACE OF STERILE, UNSALTED BUTTER

Culture	Extent of growth at 10–12° C., after									
	1 week		2 weeks		3 weeks		4 weeks		6 weeks	
	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid
1. *Alt. humicola*	—	—	—	—	—	+G	—	+++	—	++++
2. *A. flavus*	—	—	—	—	—	—	—	—	—	—
3. *A. niger*	—	—	—	—	—	—	—	—	—	—
4. *H. cladosporioides* ..	—	—	—	—	—	+G	—	+++	—	+++
5. *M. sylvaticus*	—	—	—	—	—	—	—	+WB	—	+
6. *O. lactis var. A.* ...	—	—	—	—	—	—	—	+W	—	+
7. *O. lactis var. B.* ...	—	—	—	—	—	—	—	—	—	—
8. *P. biforme*	—	+W	—	+	—	++	—	++	—	++++WG
9. *P. expansum*	—	+W	—	+	—	++	—	++WG	—	+++
10. *R. nigricans*	—	—	—	—	—	—	—	+W	—	+
Check	—	—	—	—	—	—	—	—	—	—

TABLE XXXIII

EFFECT OF HUMIDITY UPON GROWTH OF MOLD CULTURES ON UNSALTED BUTTER CHURNED FROM INOCULATED STERILE CREAM

Culture	Extent of growth at 20 25° C., after									
	1 week		2 weeks		3 weeks		4 weeks		6 weeks	
	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid
1. *Alt. humicola*	—	—	—	—	—	—	—	—	—	—
2. *A. flavus*	—	+WY	+W	+	+	++YG	+	++	+	++
3. *A. niger*	—	+B	—	+	—	+	—	+	—	+
4. *H. cladosporioides* ..	++G	++G	+++	+++	+++	+++	+++	+++	+++	+++
5. *M. sylvaticus*	—	+W	—	++	—	++	—	++	—	++
6. *O. lactis var. A.* ...	—	—	—	—	—	—	—	—	—	—
7. *O. lactis var. B.* ...	—	—	—	—	—	—	—	—	—	—
8. *P. biforme*	—	+W	+W	+	+	+	+	+	+	+
9. *P. expansum*	—	+G	—	++	—	++	—	++	—	++
10. *R. nigricans*	—	—	—	—	—	—	—	—	—	—
Check	—	—	—	—	—	—	—	—	—	—

odor; Culture 10 developed a scanty, white aerial mycelium. There were no signs of growth with Cultures 2 and 3. It will be pointed out later that these cultures do not grow well at 10° C. and for this reason did not develop on the samples studied in this trial.

b. Effect of Humidity on the Growth of Molds on Unsalted Butter Churned from Inoculated Cream

Methods. Eleven lots of sweet, 40 per cent cream were placed in Erlenmeyer flasks and autoclaved. When they were cool, they were inoculated with the cultures being studied, except one lot which was held as a check. The inoculated creams were allowed to stand at room temperature (20° to 25° C.) for 4 days after which they were placed at 0° C. until cooled sufficiently to churn. During the 4 days, all the cultures had grown extremely well and covered the surface of the cream with a deep layer of mycelium bearing the characteristic fruiting bodies. Each lot of cream was churned in a sterile Dazey churn, the butter washed with sterile water and worked with sterile paddles under careful conditions. Pieces of the feltlike mass formed by the mycelium were removed as far as possible from the butter. Small blocks of the butter were placed in petri plates. One cc. of water was placed in each of the plates of one set of samples and replenished daily, while the other set was left without water. Both lots were incubated at 20° to 25° C. for 6 weeks.

Results. Table XXXIII shows that some of the samples maintained at low humidities showed the growth of mold after one or 2 weeks but in most cases the development on the samples at higher humidities gave evidence of more favorable growing conditions. Culture 4 became clearly visible after one week under both conditions, and produced a very dark green smudge and surface spots. A delicate white web of mycelium was formed by Culture 2 on the dry samples, but bright yellowish-green sporangia on the butter well supplied with moisture. Culture 8 produced white spots under both conditions. There were no visible signs of growth in Cultures 1, 6, 7, and 10.

C. Temperature

That temperature has a profound effect upon the growth of microörganisms is generally appreciated. Each species has its minimum, optimum, and maximum temperatures, which vary with the conditions under which the organisms are existing. Butter is kept at a wide range of storage temperatures but these are, in most cases, near 0° C. or below. Occasionally, lots of butter will be exposed to higher temperatures for considerable periods of time. The ten species of molds used in these experiments were seeded on various substrata in order to observe the effect of different temperatures upon their growth. The

temperatures used did not go below 0° C. but subsequent studies should be pursued at lower temperatures for extended periods.

1. Growth on Whey Broth

Methods. Fresh skimmilk was heated to 35° C. and maintained at that temperature while dilute hydrochloric acid was added slowly, with constant stirring. The milk was brought to the isoelectric point of casein, pH 4.6 to 4.7 by comparison with methyl red standards. The finely granulated casein was removed by filtering through cheesecloth. The resultant whey was autoclaved for 15 minutes to coagulate the heat-labile proteins and filtered through cotton. The whey was neutralized with N/1 sodium hydroxide to pH 6.8, and 0.5 per cent Bacto peptone was added. The mixture was autoclaved for 15 minutes to precipitate the acid-soluble substances and filtered through paper, after which it was tubed and sterilized. Three sets of inoculated samples were prepared. One set was stored at 20° to 25° C. and 40 to 50 per cent relative humidity, another set at 10° to 12° C. and 70 to 75 per cent humidity, and the other at 0° to 2° C. and 70 to 75 per cent humidity.

Results. Table XXXIV gives the results of this experiment. It is evident that all the cultures found the whey broth an excellent source of food, as shown by the active growth at 20° C. after one week. A certain amount of subsurface mycelium was present in most cases, especially after 3 weeks at this temperature. Culture 2 was very slow to develop at 10° C. and when it did the mycelium was scanty. Culture 3 likewise made slight progress at this temperature. The growth of all cultures was retarded when the whey broth was kept at 0° C. Cultures 2, 3, and 10 gave no evidence of growth within 3 weeks. Cultures 1 and 5 produced traces of visible mycelium in the depths of the liquids. Time was an apparent factor at the lower temperatures, altho certain of the species showed no growth after longer periods of incubation.

2. Growth on Whey Agar Slants

Methods. A solid medium containing 1.2 per cent Bacto agar was prepared from whey broth made according to the method outlined in the previous experiment. This medium was tubed, autoclaved, and slanted. After inoculation, the three sets of samples were stored under the conditions described in the foregoing trial.

Results. All the cultures grew luxuriantly after one week at 20° C., as indicated in Table XXXV. Except Culture 2, which failed to grow, and Culture 3 which developed poorly, all showed practically normal growth at 10° C. At 0° C. the development of cultures that grew at all was less luxuriant. Cultures 2, 3, and 10 failed to produce

TABLE XXXIV

EFFECT OF TEMPERATURE UPON GROWTH OF MOLD CULTURES IN WHEY BROTH

Culture	Extent of growth at ordinary humidity, after								
	1 week			2 weeks			3 weeks		
	20° C.	10° C.	0° C.	20° C.	10° C.	0° C.	20° C.	10° C.	0° C.
1. *Alt. humicola*	++++(++)	+(++)	−(+)	++++(+)	+(++)	−(+)	++++(++)	++(++)	−(+)
2. *A. flavus*	++++(++)	−	−	++++(++)	−	−	++++(++)	+	−
3. *A. niger*	++++	+	−	++++	+(+)	−	++++(++)	+(+)	−
4. *H. cladosporioides* .	++++	+++(+)	+	++++	+++(+)	++(+)	++++	+++(+)	++(+)
5. *M. sylvaticus*	+++(++)	++(++)	−	+++(++)	++(++)	−(+)	+++(++)	++(++)	−(++)
6. *O. lactis var. A.* ..	++++	+++(+)	+(+)	+++(+)	+++(+)	+(+)	++++	+++(+)	+(+)
7. *O. lactis var. B.* ..	+++(++)	+++(++)	−(+)	+++(++)	+++(++)	−(+)	++++(+)	++(+)	+(+)
8. *P. biforme*	++++(+)	+++(+)	+	+++(+)	+++(+)	+(+)	++++(+)	++++(+)	+(+)
9. *P. expansum*	+++(+)	+++(+)	−	+++(+)	+++(+)	−(+)	++++(+)	+++(+)	+(+)
10. *R. nigricans*	++++(++)	++(++)	−	++++(++)	+++(+)	−	++++(++)	+++(+)	−
Check	−	−	−	−	−	−	−	−	−

TABLE XXXV

EFFECT OF TEMPERATURE UPON GROWTH OF MOLD CULTURES ON WHEY AGAR

Culture	Extent of growth at ordinary humidity, after								
	1 week			2 weeks			3 weeks		
	20° C.	10° C.	0° C.	20° C.	10° C.	0° C.	20° C.	10° C.	0° C.
1. *Alt. humicola*	++++	+++	+	++++	++++	+	++++	++++	++
2. *A. flavus*	++++	−	−	++++	−	−	++++	−	−
3. *A. niger*	+++	+	−	+++	+	−	+++	+	−
4. *H. cladosporioides*	++++	++++	+	++++	++++	++	++++	++++	++
5. *M. sylvaticus*	+++	++	+	++++	++	+	++++	++	++
6. *O. lactis var. A.*	+++	+++	+	++++	+++	+	++++	+++	++
7. *O. lactis var. B.*	+++	+++	+	++++	+++	+	++++	+++	+
8. *P. biforme*	++++	++++	++	++++	++++	++	++++	++++	+++
9. *P. expansum*	++++	+++	+	++++	+++	+	++++	+++	+
10. *R. nigricans*	++++	++++	−	++++	++++	−	++++	++++	−
Check	−	−	−	−	−	−	−	−	−

visible mycelium. Low temperatures had a marked effect on the growth, especially with Cultures 2 and 3.

3. Growth in Sweet Buttermilk

Methods. Buttermilk was obtained from a churning of fresh, sweet cream, tubed, and autoclaved. The inoculated samples were divided into three lots and incubated under the conditions explained in the last two experiments.

Results. Table XXXVI shows that the cultures kept at 20° C. did not develop as promptly in the buttermilk as they did in whey broth or whey agar. Growth at the end of 3 weeks was reasonably good in all cases. The development at 10° C. was somewhat less in most instances. Culture 2 showed no visible growth, Culture 3 gave barely visible growth after 3 weeks. Cultures 2, 3, and 10 again failed to grow at 0° C. during the observation period. In general, the surface growth on buttermilk was somewhat less than it had been on whey broth and whey agar.

4. Growth on Sterile Butter

Methods. A batch of sweet cream was autoclaved and, after cooling, churned in a sterile Dazey churn. The butter was washed with sterile water and worked with sterile paddles. The finished butter was divided into four portions, one of which was left unsalted. Sterile salt was worked into the other lots of butter in varying amounts, giving a final salt content (by analysis) of 1.2 per cent, 2.6 per cent, and 2.9 per cent, respectively. Blocks of each lot of butter were placed in petri plates and allowed to harden over night at 0° C. The samples were inoculated by streaking the cultures across the surface. One set of samples was incubated at 0° C. and the other at 10° C. in the metal humidors, at a relative humidity of 100 per cent.

Results. The observations made on the samples over a period of 6 weeks are reported in Table XXXVII. At 0° C., Culture 9 showed growth after 2 weeks, Culture 1 after 4 weeks, and Culture 8 after 6 weeks in the unsalted butter; none of the others showed any apparent development even after 6 weeks. Cultures 1 and 8 produced small white spots at this temperature in the butter containing 1.2 per cent salt. Otherwise, growth of all cultures was retarded by the combined influence of low temperature and salt. At 10° C. growth in the unsalted butter was extensive in most instances and after 6 weeks, Cultures 1 and 4 produced dark green smudges, Cultures 5 and 10 developed slight aerial mycelia, Culture 6 showed a small amount of white mycelium, and Cultures 8 and 9 formed white and green spots. Cultures 2 and 3 were not able to grow. In the butter containing 1.2 per cent of salt, and kept at 10° C., Culture 5 produced a few aerial

TABLE XXXVI

EFFECT OF TEMPERATURE UPON GROWTH OF MOLD CULTURES IN SWEET BUTTERMILK

Culture	Extent of growth at ordinary humidity, after								
	1 week			2 weeks			3 weeks		
	20° C.	10° C.	0° C.	20° C.	10° C.	0° C.	20° C.	10° C.	0° C.
1. *Alt. humicola*	+	++	—	++	++	—	+++	+++	+
2. *A. flavus*	+++	—	—	+++	—	—	++++	—	—
3. *A. niger*	++	—	—	+++	—	—	++++	+	—
4. *H. cladosporioides*	++++	++	—	++++	++	—	++++	+++	++
5. *M. sylvaticus*	+++	+++	++	+++	+++	++	++++	+++	++
6. *O. lactis var. A.*	++++	++	++	++++	++	++	++++	++	++
7. *O. lactis var. B.*	++++	++	++	++++	++	++	++++	++	++
8. *P. biforme*	+++	+++	+++	+++	+++	+++	+++	+++	+++
9. *P. expansum*	+++	++	++	+++	++	++	+++	+++	++
10. *R. nigricans*	++++	+	—	++++	++	—	++++	++	—
Check	—	—	—	—	—	—	—	—	—

TABLE XXXVII

EFFECT OF TEMPERATURE UPON GROWTH OF MOLD CULTURES ON STERILE BUTTER

Culture	Extent of growth, at high humidities, after 6 weeks							
	Unsalted		1.2 per cent salt		2.6 per cent salt		2.9 per cent salt	
	10° C.	0° C.	10° C.	0° C.	10° C.	0° C.	10° C.	0° C.
1. *Alt. humicola*	++++G	+W	++G	+W	+G	—	+WG	—
2. *A. flavus*	—	—	—	—	—	—	—	—
3. *A. niger*	—	—	—	—	—	—	—	—
4. *H. cladosporioides*	+++G	—	++G	—	+G	—	+G	—
5. *M. sylvaticus*	+W	—	+W	—	—	—	—	—
6. *O. lactis var. A.*	+W	—	—	—	—	—	—	—
7. *O. lactis var. B.*	—	—	—	—	—	—	—	—
8. *P. biforme*	++++WG	+W	++WG	+G	++G	—	++WG	—
9. *P. expansum*	+++WG	+G	++G	—	++G	—	++G	—
10. *R. nigricans*	+W	—	—	—	—	—	—	—
Check	—	—	—	—	—	—	—	—

Note: The same series of samples, maintained at the same temperatures, but at low humidity, failed to show any evidence of growth during 6 weeks.

hyphae; Cultures 1, 4, 8, and 9 grew quite well and produced distinct areas of moldiness. The butters containing 2.6 per cent and 2.9 per cent salt showed slight growth of Cultures 1, 4, 8, and 9. The low temperatures clearly restrained the growth of most of the species studied, and the effect became more noticeable as the percentage of salt increased.

5. Growth on Butter Made from Inoculated Cream

Methods. Eleven batches of fresh sweet cream in flasks were autoclaved and cooled, after which ten lots were inoculated with the experimental cultures and the other left as a check. The inoculated cream samples were kept at 20° to 25° C. for 4 days. During this time, all cultures grew exceedingly well and produced a surface felt with normal fructification. These samples were churned in individual, sterile Dazey churns. The butter was washed with sterile water and worked with sterile paddles. Visible pieces of the felt were removed, as far as possible. Each lot of butter was divided into four parts—one remained unsalted, the others were worked with different amounts of sterile salt in order to obtain varying concentrations. The actual salt content in the different lots of butter varied as indicated in Table XXXVIII. It was very difficult to obtain exactly the same results in each case, because of the difficulty of working the butter under aseptic conditions. Blocks of the butters were placed in petri plates and hardened at 0° C. One set of plates was placed at 20° to 25° C., another at 10° to 12° C., and another at 0° to 2° C. in the metal humidors at a relative humidity of 100 per cent and observations were made during 6 weeks.

Results. The combined effect of temperature and salt concentration on the growth of the various species is shown in Table XXXVIII. It will be noted that Cultures 1, 6, 7, and 10 failed to make any visible growth under any conditions. Cultures 2 and 3 grew reasonably well at 20° C. in all salt concentrations but apparently could not develop at 10° or 0° C. Culture 4 grew at all three temperatures but was affected by increased percentages of salt, especially at 0° C. The only visible growth secured with Culture 5 was on the unsalted sample at 20° C. Results with Culture 8 were irregular, but it grew at all temperatures. With the higher salt concentrations and lower temperatures its development was retarded or checked. Culture 9 was not affected particularly by the salt content of the butter but failed to appear at 0° C. Evidently, the effect of temperature varied with the species of mold and the composition of the substrate as influenced by the salt content.

D. Atmosphere

Most species of molds are considered aerobic, or at least facultative, and this indicates that a satisfactory supply of oxygen is a factor

TABLE XXXVIII

EFFECT OF TEMPERATURE UPON GROWTH OF MOLD CULTURES IN BUTTER FROM INOCULATED CREAM

Culture	Per cent of salt	Extent of growth, high humidity, after								
		2 weeks			4 weeks			6 weeks		
		20° C.	10° C.	0° C.	20° C.	10° C.	0° C.	20° C.	10° C.	0° C.
1. *Alt. humicola*	0	—	—	—	—	—	—	—	—	—
	1.5	—	—	—	—	—	—	—	—	—
	2.7	—	—			—		—	—	—
	4.2	—	—	—	—	—	—			—
2. *A. flavus*	0	+		—	++	—	—	++	—	—
	1.4	++		—	+++	—	—	+++	—	—
	2.2	+++		—	+++	—	—	+++	—	—
	4.0	++		—	++	—	—	++	—	—
3. *A. niger*	0	+		—	+	—	—	+		—
	1.5	++	—	—	+++	—	—	+++	—	—
	2.5	++	—	—	++	—	—	++	—	—
	4.5	++		—	++	—	—	++	—	—
4. *H. cladosporioides* ..	0	+++	++	+	+++	+++	++	+++	++++	++
	1.6	++	++	—	++	+	+	++	+	+
	2.7	++	+	—	+	+	—	+	+	—
	3.3	+	++	—	+	+	—	+	+	—
5. *M. sylvaticus*	0	++	—	—	++	—	—	++	—	—
	1.1	—	—	—	—	—	—	—		—
	2.3	—	—	—	—	—	—	—	—	—
	3.5	—	—	—	—	—	—	—	—	—
6. *O. lactis var. A.* ...	0		—	—	—	—	—	—		
	1.4	—	—	—	—	—	—		—	—
	1.6	—	—	—	—	—	—	—	—	—
	3.0	—	—	—	—	—	—	—	—	—
7. *O. lactis var. B.* ...	0	—	—	—		—	—	—	—	—
	0.9	—	—	—	—	—	—	—		
	2.1	—	—	—		—		—	—	—
	3.1	—	—	—	—	—	—	—	—	
8. *P. biforme*	0	+	+	+	+	++	++	+	++	++
	0.7	—	+	—	—	++	—	—	++	+
	2.3	+	—	—	+	++		+	++	—
	3.1	+	—	—	+	—	—	+		—
9. *P. expansum*	0	++	+	—	++	++	—	++	++	—
	1.3	++	+	—	++	++	—	++	++	—
	2.2	++	+	—	++	++	—	++	++	—
	3.0	+	+	—	+	++	—	+	++	—
10. *R. nigricans*	0	—		—		—	—	—	—	
	1.3	—	—	—	—			—	—	—
	2.1	—	—	—	—	—	—	—	—	—
	4.0	—	—	—	—	—	—	—	—	—
Check	0.0	—	—	—	—	—	—	—	—	—

in their growth. Experiments were undertaken to determine how the mold growth on butter might be affected by changes in atmospheric conditions.

1. Ordinary Air Supply

In the experiments previously reported, and in which a plentiful supply of oxygen was provided, the species of molds making up the experimental group were able to grow satisfactorily when the food supply, moisture, temperature, and other conditions were favorable. These results may be considered as checks demonstrating that the molds grew well when an abundant supply of air was present.

2. Reduced Air Supply—Partial Vacuum

Methods. Sterile cream was churned in a sterile Dazey churn. The butter was washed with sterile water and worked with sterile paddles. Blocks of butter were placed in petri plates (in which the covers were kept slightly raised by the use of small wire staples) and hardened at 0° C. The surface of the butter was inoculated with the molds being studied. The plates were then placed in an ordinary sterile glass desiccator. After a liter of water was placed in the the bottom of the jar to provide adequate humidity, the lid was put in place and sealed with vaseline. A vacuum of 25 inches was drawn and maintained during the period of study. The temperature of incubation was 20° to 25° C.

Results. The results of the trial are given in Table XXXIX. Growth was not very active during the first week but increased somewhat during the subsequent 3 weeks. The development was never very great, however, and was not comparable with that obtained under ordinary atmospheric conditions. The reduction of the amount of available oxygen appeared to retard the development of these species.

TABLE XXXIX

Effect of Reduced Air Supply upon Growth of Mold Cultures on Sterile, Unsalted Butter

Extent of growth, at 20–25° C., high humidity, under 25 inches of vacum, after	Culture No.*										
	1	2	3	4	5	6	7	8	9	10	Check
1 week	+	+	+	+	−	−	−	+	−	−	−
4 weeks	+	++	++	++	+	−	+	++	+++	+	−

* See Table XXXVIII for species.

3. Partial Removal of Carbon Dioxide

Methods. The butter used was prepared in the same manner as that for 2, above. The plates were held in a sterile desiccator in the bottom of which was placed a liter of a 10 per cent aqueous solution of sodium hydroxide. This was added to absorb some of the carbon

dioxide; at the same time it was a source of moisture. The lid of the desiccator was sealed with vaseline. The temperature of incubation was 20° to 25° C.

TABLE XL

EFFECT OF PARTIAL REMOVAL OF CARBON DIOXIDE FROM ATMOSPHERE UPON GROWTH MOLD CULTURES ON STERILE, UNSALTED BUTTER

Extent of growth at 20–25° C., high humidity, (10% soln. of NaOH) in desiccator, after	Culture No.*										
	1	2	3	4	5	6	7	8	9	10	Check
1 week	+	+	+	+	+	+	+	++	++	++	—
4 weeks	+++	++	+++	+++	++++	+	+	+++	+++	++++	—

* See Table XXXVIII for species.

Results. Altho the growth of the cultures was not extensive during the first week, Table XL shows that excellent development of all except Cultures 6 and 7 occurred after 4 weeks. However, the last-named cultures actually showed more visible growth under these conditions than they had shown at any other time when seeded on butter. Apparently, a reduction in the amount of carbon dioxide does not seriously deter the growth of the species under observation.

4. Removal of Oxygen

Methods. The butter used was prepared in the manner described in 2 and 3, above. The plates were placed in a sterile desiccator and the lid sealed with vaseline, after 100 grams of pyrogallic acid had been placed in the bottom. A funnel was introduced through an opening in the side of the desiccator and 500 cc. of a 10 per cent aqueous solution of sodium hydroxide were added to the pyrogallic acid. The funnel was removed quickly and the opening closed and sealed. The temperature of incubation was 20° to 25° C.

TABLE XLI

EFFECT OF REMOVAL OF OXYGEN FROM THE ATMOSPHERE UPON THE GROWTH OF MOLD CULTURES ON STERILE, UNSALTED BUTTER

(Oxygen exhausted by NaOH–pyrogallic acid mixture)

Extent of growth at 20–25° C., high humidity, after	Culture No.*										
	1	2	3	4	5	6	7	8	9	10	Check
1 week	—	—	—	—	—	—	—	—	—		
4 weeks	—	—	—	—							—

* See Table XXXVIII for species.

Results. Table XLI points out clearly that no growth appeared in any of the samples even after 4 weeks. The exhaustion of oxygen apparently made conditions unfavorable for the development of the molds being studied.

E. Miscellaneous Chemical and Physical Factors

1. Salt Content

Sodium chloride has been considered a food preservative for many years. As most butter contains some salt, and as unsalted butter has appeared to be more susceptible to molding than the salted type, experiments were conducted to determine the effect of various concentrations of sodium chloride upon the growth of the species of molds selected for this study.

a. Whey Broth Plus Salt

Methods. Whey broth prepared in the manner described in previous experiments was used as a substrate. Sodium chloride was added to various portions in different amounts, so the final salt content (by weight) in the different lots of broth was 10, 15, and 20 per cent. An unsalted portion was left as a check. The solutions were tubed and autoclaved. After inoculation the samples were placed at 20° to 25° C. in a room with a relative humidity of 40 to 50 per cent, and observed for 3 weeks.

Results. The record of the observations is given in Table XLII. The growth of all the cultures was excellent in the unsalted broth, especially on the surface. In the broth containing 10 per cent of salt, Cultures 2, 3, 4, 8, and 9 had produced abundant mycelium and fructification at the end of 3 weeks. Cultures 1, 5, and 10 produced only subsurface growth; Cultures 6 and 7 were checked entirely. In the 15 per cent salt broth, Cultures 2, 8, and 9 grew fairly well on the surface; Cultures 1, 3, and 4 showed some subsurface mycelium. The remaining cultures gave no growth with 20 per cent salt. The only species to show any development in this broth were Cultures 8 and 9 and they grew well. It is evident that salt exercised a restraining effect upon the growth of molds but it varied considerably with the different species.

b. Whey Agar Plus Salt

Methods. The whey agar used in this experiment was similar to that described previously. Sodium chloride was added to portions of the medium so that the final concentrations by weight were 10, 15, and 20 per cent. These and the unsalted check were tubed, autoclaved, and slanted. After inoculation they were incubated under conditions similar to those in the preceding experiment.

Results. All cultures developed luxuriantly on the unsalted slants, as reported in Table XLIII. In the 10 per cent salt agar, Cultures 6 and 7 were the only ones that failed to grow, altho Cultures 5 and 10 showed very meager growth. When the salt content reached 15 per cent, Cultures 2, 8, and 9 showed moderate growth and Cultures

TABLE XLII

EFFECT OF SALT UPON GROWTH OF MOLD CULTURES ON WHEY BROTH

Culture	Extent of growth at 20–25° C., ordinary humidity, after											
	1 week				2 weeks				3 weeks			
	Per cent of salt				Per cent of salt				Per cent of salt			
	0	10	15	20	0	10	15	20	0	10	15	20
1. *Alt. humicola*	++++(++)	−(+)	−	−	++++(++)	−(++)	−	−	++++(++)	−(++)	−(+)	−
2. *A. flavus*	++++(++)	+++	++	−	++++(++)	+++(+)	++(+)	−	++++	+++(+)	++(+)	−
3. *A. niger*	++++	+++(+)	−	−	++++	+++(+)	−(++)	−	++++(++)	+++(+)	−(++)	−
4. *H. cladosporioides*	++++	+++(+)	−	−	++++	+++(+)	−	−	++++	+++(+)	−(+)	−
5. *M. sylvaticus*	+++(++)	−(+)	−	−	+++(++)	−(+)	−	−	+++(++)	−(+)	−	−
6. *O. lactis var. A.*	++++	−	−	−	++++	−	−	−	++++	−	−	−
7. *O. lactis var. B.*	+++(++)	−	−	−	++++(++)	−	−	−	++++(++)	−	−	−
8. *P. biforme*	++++(+)	+++(+)	++	−	++++(+)	+++(+)	+++(+)	+	++++(++)	+++(+)	++(+)	++
9. *P. expansum*	+++(+)	+++(+)	++	+	++++(+)	+++(+)	++(+)	++	++++(+)	+++(+)	++(+)	++
10. *R. nigricans*	++++(++)	−	−	−	++++(++)	−(+)	−	−	++++(++)	−	−	−
Check	−	−	−	−	−	−	−	−	−	−	−	−

TABLE XLIII

EFFECT OF SALT UPON GROWTH OF MOLD CULTURES ON WHEY AGAR

Culture	Extent of growth at 20–25° C., ordinary humidity, after											
	1 week				2 weeks				3 weeks			
	Per cent of salt				Per cent of salt				Per cent of salt			
	0	10	15	20	0	10	15	20	0	10	15	20
1. *Alt. humicola*	++++	++	−	−	++++	+++	+	−	++++	+++	+	−
2. *A. flavus*	++++	+++	++	−	++++	+++	++	−	++++	+++	++	−
3. *A. niger*	+++	+++	+	−	+++	++	+	−	+++	++	+	−
4. *H. cladosporioides*	++++	+++	−	−	++++	+++	+	−	++++	+++	++	−
5. *M. sylvaticus*	+++	+	−	−	+++	+	−	−	+++	+	−	−
6. *O. lactis var. A.*	+++	−	−	−	+++	−	−	−	+++	−	−	−
7. *O. lactis var. B.*	+++	−	−	−	+++	−	−	−	+++	−	−	−
8. *P. biforme*	++++	++++	++	+	++++	+++	++	+	++++	+++	++	+
9. *P. expansum*	++++	++	++	+	++++	++	++	+	++++	++	++	+
10. *R. nigricans*	++++	+	−	−	++++	+	−	−	++++	+	−	−
Check	−	−	−	−	−	−	−	−	−	−	−	−

1 and 4 slight growth. The others remained dormant. As in the previous experiment Cultures 8 and 9 were the only ones to grow in the 20 per cent concentration of salt and their growth was scanty. Growth was better on the solid medium than on the whey broth, but the influence of the salt was demonstrated to be in the same general direction.

c. Sterile, Sweet Buttermilk Plus Salt

Methods. Buttermilk was obtained directly from a churning of sweet cream. The necessary salt was added to yield solutions containing 5, 10, 15, and 20 per cent. An unsalted portion was kept as a check. All lots were tubed and autoclaved. After inoculation they were incubated under the conditions described under "a" and "b," above.

Results. The growth of every culture was luxuriant in the unsalted buttermilk after two weeks, as is shown in Table XLIV. In the 5 per cent buttermilk, development was excellent after 2 weeks in the case of Cultures 1, 2, 3, 4, 8, and 9, moderately good with Cultures 5 and 10, but only slight with Cultures 6 and 7. The 10 per cent salt concentration completely inhibited Cultures 5, 6, 7, and 10, but the rest grew well. When the percentage of salt was increased to 15 per cent, only Cultures 2, 3, 8, and 9 were active. With 20 per cent salt, only Culture 9 produced visible growth. It will be noted that the growth of certain species is retarded more in the salted buttermilk than in whey broth or agar.

d. Sterile, Sour Buttermilk Plus Salt

Methods. A sample of buttermilk was obtained from a churning of sour cream butter. Salt was added to portions of this buttermilk to give concentrations of 5, 10, 15, 20, and 25 per cent, and an unsalted check was retained. These solutions were tubed and autoclaved. After inoculation, they were incubated at 20° to 25° C. for 2 weeks at a relative humidity of 60 to 65 per cent.

Results. Table XLV presents the results obtained in this experiment. In the unsalted buttermilk the growth after 2 weeks was luxuriant but it had been slower than that obtained on the sweet buttermilk. The development of the cultures in the 5 per cent salt solution was practically the same as in the unsalted samples, in some cases less and in other cases greater. In the buttermilk containing 10 per cent of salt, Cultures 2, 3, 4, 8, and 9 grew well. When the concentration of salt was increased to 15 per cent, Cultures 2, 3, 8, and 9 were the only ones to produce visible mycelium and fruiting bodies. The 20 per cent solution retarded all except Culture 9, and in a 25 per cent concentration of salt none were able to grow during the 2 weeks incubation period.

TABLE XLIV

EFFECT OF SALT UPON GROWTH OF MOLD CULTURES IN SWEET BUTTERMILK

Culture	Extent of growth at 20–25° C., ordinary humidity, after 4 days, Per cent of salt					Extent of growth at 20–25° C., ordinary humidity, after 2 weeks, Per cent of salt				
	0	5	10	15	20	0	5	10	15	20
1. *Alt. humicola*	+++W	+W	—	—	—	+++G	+++WG	++G	—	—
2. *A. flavus*	++WY	++WY	++Y	—	—	+++YG	+++YG	+++YG	+W	—
3. *A. niger*	++WBr	+WBr	+Br	—	—	+++Br	+++Br	++Br	+W	—
4. *H. cladosporioides*	+++G	++G	+G	—	—	+++G	+++G	++G	—	—
5. *M. sylvaticus*	++W	+W	—	—	—	+++WB	++WB	—	—	—
6. *O. lactis var. A.*	++W	—	—	—	—	+++W	+W	—	—	—
7. *O. lactis var. B.*	+++W	—	—	—	—	+++W	+W	—	—	—
8. *P. biforme*	++W	+W	+W	—	—	+++G	+++G	++W	+W	—
9. *P. expansum*	+++G	++G	+WG	+WG	—	+++G	+++G	+++G	++WG	+WG
10. *R. nigricans*	+++WB	+W	—	—	—	+++WB	++WB	—	—	—
Check	—	—	—	—	—	—	—	—	—	—

TABLE XLV

EFFECT OF SALT UPON GROWTH OF MOLD CULTURES IN SOUR BUTTERMILK

Culture	Extent of growth at 20–25° C., ordinary humidity, after 3 days, Per cent of salt						Extent of growth at 20–25° C., ordinary humidity, after 2 weeks, Per cent of salt					
	0	5	10	15	20	25	0	5	10	15	20	25
1. *Alt. humicola*	++W	+W	+W	—	—	—	+++WG	++G	+W	—	—	—
2. *A. flavus*	++YG	+Y	+Y	—	—	—	+++YG	+++YG	+++YG	+YG	—	—
3. *A. niger*	+WB	+B	+B	—	—	—	++B	+++B	+++B	+Br	—	—
4. *H. cladosporioides*	+G	+G	—	—	—	—	+++G	+++G	++G	—	—	—
5. *M. sylvaticus*	++W	+W	—	—	—	—	+++WB	++WB	—	—	—	—
6. *O. lactis var. A.*	++W	+W	—	—	—	—	+++WY	++WY	—	—	—	—
7. *O. lactis var. B.*	++W	+W	—	—	—	—	+++WY	++WY	—	—	—	—
8. *P. biforme*	+W	+W	+W	+W	—	—	+++G	++G	+++WY	+G	—	—
9. *P. expansum*	++WG	+WG	+W	—	—	—	+++G	+++G	+++G	++G	+W	—
10. *R. nigricans*	++W	+W	—	—	—	—	+++WB	++WB	—	—	—	—
Check	—		—	—	—	—	—	—	—	—	—	—

In order to determine whether the cultures that had failed to grow on the buttermilk containing 15 per cent of salt were simply retarded or actually killed, a portion of each sample was plated on whey agar. Normal colonies were obtained from all such platings, proving that the spores were still viable but unable to grow in the strong brine. In a similar way, when samples were taken from the 25 per cent solution, it was found that Cultures 2, 3, 5, 8, 9, and 10 had been unharmed by the salt while Cultures 1, 4, 6, and 7 apparently had been destroyed, as no colonies were obtained. Accordingly, it appears that moderately strong brines retard the growth of certain species; higher concentrations may actually destroy some species.

e. Sterile Butter Plus Salt

Methods. A batch of sweet cream was autoclaved, cooled, churned in a sterile Dazey churn, and the butter washed with sterile water. The butter was divided into five lots, one of which was retained without salt as a check. Sterilized salt was added in varying amounts to the other four lots. All were worked as thoroly and carefully as possible with sterile paddles. The salt contents of the four lots of salted butter, as determined by analysis, were 0.5, 0.8, 1.4, and 1.7 per cent, respectively. Small blocks of each were placed in petri plates and hardened at 0° C. over night. The next day they were inoculated by streaking the cultures across the surface. The intention was to incubate these samples at 5° to 6° C. at a high humidity for 6 weeks, but circumstances made it necessary to remove the plates from the humidor after one week. During the next 2 weeks they were kept at 5° to 6° C. but at a lower humidity. At the end of 3 weeks it was necessary to remove the samples from the cooler. For the following 3 weeks they were kept at 20° to 25° C. at a high humidity. Consequently, conditions were so variable that results are not very satisfactory. Analyses of the butters showed the following compositions:

	Per cent				
Salt	0.0	0.5	0.8	1.4	1.7
Water	14.0	14.4	14.2	14.2	14.8
Salt in brine	0.0	3.4	5.3	8.9	10.3

This makes it possible to interpret the results in terms of those obtained with buttermilk, whey broth etc.

Results. The results of this trial are recorded in Table XLVI. No growth was visible in any of the samples held at 6° C. during the 2 weeks, except Culture 9, which had produced a small white spot in the unsalted butter. After 3 weeks, Cultures 1, 4, and 9 had developed visible colored areas in the unsalted butter. The following week witnessed the growth of Cultures 3 and 10 in addition to those mentioned. After

TABLE XLVI

Effect of Salt upon Growth of Mold Cultures on Sterile Butter

Culture	Extent of growth, 1st week at 6° C., high humidity; 2nd and 3rd weeks at 6° C., ordinary humidity; 4th, 5th, and 6th weeks at 20–25° C., high humidity, after														
	1 week					3 weeks					6 weeks				
	Per cent of salt					Per cent of salt					Per cent of salt				
	0	0.5	0.8	1.4	1.7	0	0.5	0.8	1.4	1.7	0	0.5	0.8	1.4	1.7
1. *Alt. humicola*	—	—	—	—	—	+WG	—	—	—	—	+++G	++G	++G	++G	+G
2. *A. flavus*		—	—	—	—	—	—	—	—	—	+W	++Y	+Y	+Y	++YG
3. *A. niger*	—	—	—	—	—	—	—	—	—	—	+++B	+++B	++B	++B	++B
4. *H. cladosporioides*	—	—	—	—	—	+G	—	—	—	—	+++G	+++G	++G	++G	++G
5. *M. sylvaticus*	—	—	—	—	—	—	—	—	—	—	++WB	++W	—	—	—
6. *O. lactis var. A.*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7. *O. lactis var. B.*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8. *P. biforme*	—	—	—	—	—	—	—	—	—	—	+W	+G	+W	+W	+W
9. *P. expansum*	—	—	—	—	—	+W	—	—	—	—	+++G	+++G	+++G	+++G	+++G
10. *R. nigricans*	—	—	—	—	—	—	—	—	—	—	++W	++W	—	—	—
Check	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

TABLE LXVII

Effect of Salt upon Growth of Mold Cultures on Sterile Butter

Culture	Extent of growth at 10° C., high humidity, after												
	1 week				3 weeks				6 weeks				
	Per cent of salt				Per cent of salt				Per cent of salt				
	0	1.2	2.6	2.9	0	1.2	2.6	2.9	0	1.2	2.6	2.9
1. *Alt. humicola*	—	—	—	—	+G	—	—	—	++++G	++G	+G	+WG
2. *A. flavus*	—	—	—	—	—	—	—	—	—	—	—	—
3. *A. niger*	—	—	—	—	—	—	—	—	—	—	—	—
4. *H. cladosporioides*	—	—	—	—	+G	—	—	—	+++G	++G	+G	+G
5. *M. sylvaticus*	—	—	—	—	—	—	—	—	+W	+W	—	—
6. *O. lactis var. A.*	—	—	—	—	—	—	—	—	—	—	—	—
7. *O. lactis var. B.*	—	—	—	—	—	—	—	—	—	—	—	—
8. *P. biforme*	+W	—	—	—	++W	+W	—	—	++++WG	++WG	++G	++WG
9. *P. expansum*	+W	—	—	—	++W	+W	—	—	+++WG	++G	++G	++G
10. *R. nigricans*	—	—	—	—	—	—	—	—	+W	—	—	—
Check	—	—	—	—	—	—	—	—	—	—	—	—

2 weeks at room temperature active development was observed for all cultures except 6 and 7. At the end of 6 weeks, Cultures 1, 2, 3, 4, 8, and 9 had shown some growth in all samples, salted and unsalted, but greatest in the latter. Cultures 5 and 10 were checked by the salt in the butter containing 0.8 per cent or more. Cultures 6 and 7 did not grow at all. When it is realized that the highest salt concentration on the basis of brine was 10.3 per cent in the butter containing 1.7 per cent of salt, it is not surprising that some species were able to grow, as the same cultures had demonstrated their ability to do so in buttermilk containing an equivalent amount of salt. The checking of the growth of Cultures 5 and 10 in butter with a percentage of salt in brine of more than 5.3 per cent also is in accordance with the results obtained in buttermilk. These facts emphasize the importance of considering the salt content of butter in terms of the percentage of salt in brine when it is to be taken into account as a preservative or deterrent of mold growth.

f. Sterile Butter Plus Salt

Methods. Sweet cream was autoclaved, cooled, churned in a Dazey churn, and the butter washed with sterile water. The butter was divided into four lots, one of which was left unsalted to serve as a check. Sterile salt was added to the three portions in amounts sufficient to give final salt contents of 1.2, 2.6, and 2.9 per cent, respectively. Each lot of butter was worked separately with sterile paddles and under aseptic conditions. Blocks of each butter were placed in petri plates and held at 0° C. over night for hardening. The samples were inoculated on the surface and placed in the metal humidor and stored at 10° C. for 6 weeks at a relative humidity of 100 per cent.

The composition of these four lots of butter as determined by analysis was as follows:

	Per cent			
Salt	0.0	1.2	2.6	2.9
Water	15.9	15.6	16.0	16.0
Salt in brine	0.0	7.1	13.9	15.3

Results. The data are reported in Table XLVII. Cultures 8 and 9 were the only ones to show growth during the first 2 weeks and then only on the unsalted butter. After 3 weeks Cultures 1 and 4 grew on the unsalted butter; Cultures 8 and 9 also developed on the butter containing 1.2 per cent salt. Cultures 1, 4, 8, and 9 developed extensively in the unsalted butter after 4 weeks and Cultures 5, 6, and 10 became barely visible. In the butter containing 1.2 per cent salt, Cultures 1, 4, 8, and 9 grew slightly. Culture 1 produced a small dark green spot on the butter with 2.6 per cent salt; Cultures 8 and 9 grew

on both the 2.6 per cent and the 2.9 per cent samples. During the 6 weeks, Cultures 1, 4, 8, and 9 developed exceedingly well, but Cultures 5 and 10 set up scanty aerial mycelium, in the unsalted butter. In the butter containing 1.2 per cent salt, Cultures 1, 4, 8, and 9 grew extensively, and Culture 5 sparsely. On the butters with the higher salt concentrations, Cultures 1, 4, 8, and 9 produced fairly distinct discolorations. These results are more or less in accordance with previous observations of the growth of these molds on media containing salt, when the salt content is considered from the standpoint of its concentration in the brine. The failure of Cultures 6 and 7 to show visible growth even in unsalted butter has been the rule, altho evidence leads to the belief that they actually may be growing even tho a visible mycelium does not appear. The explanation of the failure of Cultures 2 and 3 to develop may be found in the temperature of incubation. Previous observations demonstrated that these species did not grow well in the most favorable substrate at temperatures as low as 10° C., also that a set of samples similar to those considered in Table XLVII was kept at the same temperature but at a relative humidity of 70 to 75 per cent. These samples gave no sign of growth after 3 weeks.

g. Butter Made from Inoculated Cream Plus Salt

Methods. The butter was made from sterile cream inoculated and handled according to the procedure outlined under "b" on page 47. The butter in each case was divided into four lots and worked with varying percentages of salt. The amount of salt in each lot is indicated in Table XLVIII. Considerable difficulty was encountered in churning the cream inoculated with Culture 10. It took more than an hour, while ordinarily the churning period was 10 or 15 minutes. At the other extreme, only 2 minutes were required to churn the creams containing Cultures 2 and 3. In these three samples the water content was exceedingly high and the butter little more than a paste. The percentage of water in the various samples was as follows:

Culture	Water %
1	16.8 to 19.4
2	46.2 to 50 8
3	35.0 to 36.8
4	18.4 to 20.0
5	15.0 to 19.4
6	21.0 to 21.8
7	21.6 to 23.4
8	15.0 to 19.5
9	19.2 to 20.2
10	47.4 to 51.2

The percentage of salt in brine under these conditions is as follows:

Culture	Salt in Brine %			
1	0	7.2	13.8	18.4
2	0	2.9	4.5	7.3
3	0	3.9	5.9	11.4
4	0	7.4	12.4	15.2
5	0	9.3	12.7	15.3
6	0	6.0	6.9	12.5
7	0	4.0	8.2	12.1
8	0	4.5	11.3	13.3
9	0	6.2	10.3	12.9
10	0	2.5	4.2	7.8

The butter samples were stored for 6 weeks at a temperature of 20° to 25° C. Each plate contained one cc. of added water, which was replenished at intervals to maintain a high humidity.

Results. The results of this experiment are shown in Table XLVIII. Cultures 1, 6, 7, and 10 failed to give any visible evidence of growth, altho alterations in the aroma of the butter were marked. Culture 5 showed some development of aerial mycelium in the unsalted but was not able to grow in the salted butter. The other cultures gave irregular results that are not easy to explain, but Cultures 2, 3, 4, 8, and 9 showed a tendency to grow at all salt concentrations used; the trend being toward less growth in the most highly salted sample. Plate IV illustrates the character of the growth of Culture 4 on butters containing various amounts of salt. The unsalted sample would scarcely be recognized as butter.

DISCUSSION OF RESULTS

The data obtained in the experiments reported in the preceding pages give clues to some of the important factors influencing the growth of molds in butter. For convenience, these factors will be discussed in the order in which they were studied.

Food supply.—As pointed out in the review of literature, microörganisms must have sufficient and satisfactory nutrients if they are to develop and carry on the metabolic processes of growing cells. While certain types of organisms are able to subsist on compounds that are practically pure inorganic substances, the majority of the familiar forms require a diversity of foodstuffs. It is generally agreed that fungi seek, especially, compounds containing carbon, oxygen, hydrogen, and nitrogen, particularly in the form of organic substances. In nature, most of the materials carrying these four elements also contain others, such as phosphorus, sulphur, and a great variety of mineral elements, either intimately as a part of such compounds, or as impurities. The familiar substances commonly classed as fats, proteins, carbohydrates,

TABLE XLVIII

EFFECT OF SALT UPON GROWTH OF MOLD CULTURES IN BUTTER MADE FROM INOCULATED CREAM

Culture	Per cent of salt	Extent of growth at 20 25° C., high humidity, after					
		1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
1. *Alt. humicola* ...	0.0	—	—	—	—	—	—
	1.5	—	—	—	—	—	—
	2.7	—	—	—	—	—	—
	4.2	—	—	—	—	—	—
2. *A. flavus*	0.0	+WY	+	++YG	++	++	++
	1.4	++YG	++	+++	+++	+++	+++
	2.2	++YG	+++	+++	+++	+++	+++
	4.0	+YG	++	++	++	++	++
3. *A. niger*	0.0	+B	+	+	+	+	+
	1.5	++B	++	+++	+++	+++	+++
	2.3	++B	++	++	++	++	++
	4.5	+B	++	++	++	++	++
4. *H. cladosporioides*	0.0	++G	+++	+++	+++	+++	+++
	1.6	+G	++	++	++	++	++
	2.7	+G	++	++	++	++	++
	3.3	+G	+	+	+	+	+
5. *M. sylvaticus* ...	0.0	+W	++	++	++	++	++
	1.1	—	—	—	—	—	—
	2.3	—	—	—	—	—	—
	3.5	—	—	—	—	—	—
6. *O. lactis var. A.*	0.0	—	—	—	—	—	—
	1.4	—	—	—	—	—	—
	1.6	—	—	—	—	—	—
	3.0	—	—	—	—	—	—
7. *O. lactis var. B.*	0.0	—	—	—	—	—	—
	0.9	—	—	—	—	—	—
	2.1	—	—	—	—	—	—
	3.1	—	—	—	—	—	—
8. *P. biforme*	0.0	+W	+	+	+	+	+
	0.7	—	—	—	—	—	—
	2.3	+WG	+G	+	+	+	+
	3.1	—	+G	+	+	+	+
9. *P. expansum* ...	0.0	+G	++	++	++	++	++
	1.3	+G	++	++	++	++	++
	2.2	+G	++	++	++	++	++
	3.0	+G	+	+	+	+	+
10. *R. nigricans* ...	0.0	—	—	—	—	—	—
	1.3	—	—	—	—	—	—
	2.1	—	—	—	—	—	—
	4.0	—	—	—	—	—	—
Check	0.0	—	—	—	—	—	—

and mineral salts and the materials related to them furnish the great variety of essential elements for microbiological activity. Butter is a substance consisting principally of a mixture of fats that carry a rich supply of carbon, oxygen, and hydrogen. Held within this mass of fat are droplets of material consisting of such substances as the proteins, casein and albumin, the carbohydrate lactose, a variety of mineral salts, and by-products of the decomposition of any of these compounds, all in association with a relatively large amount of water.

It seemed desirable that a study should be made of the relative value of the various constituents of butter as foods for molds, specifically for cultures that were selected as representative of the species commonly found in butter. (See p. 22.) As fat is the principal constituent of butter, it was made the subject of the first trials. It is generally agreed that fat is not the most desirable food for microörganisms. This does not mean, however, that all forms fail to utilize the various glycerides. While it is not easy to obtain fat in an absolutely pure state, it is possible to purify it with reasonable success without altering its character appreciably. As reported in previous pages, the species of molds studied did not make significant growth on purified butterfat unless some water was present. This is reasonable, as a certain amount of water is necessary for any biological process involving the metabolism of living protoplasm. When growth was obtained on the butterfat to which water had been added, there was a possibility that it was due to other substances present as impurities in the fat. At any rate, development was not extensive under these conditions and altho germination of conidia occurred in some cases, the amount of mycelium formed was seldom great. Microscopic studies showed that in some instances initial development occurred but was soon checked and followed by a disintegration of the hyphae. The slight development on purified fats might well be attributed to a supply of nutrients within the inoculum, and present in such minute quantities that growth was soon impossible. The trials with butterfat obtained from various sources and treated in different ways did not show any particular advantage of one type of butterfat over another. None of them were to be considered as very satisfactory direct sources of food for the early development of the molds being studied, even when water was present to improve the opportunities for growth. These results are in line with previous investigations and in accordance with the observation that butter oils seldom are affected with mold growth.

Lecithin, which contains nitrogen and phosphorus in addition to carbon, hydrogen, and oxygen, is known to exist in butter. When this substance, obtained from dried buttermilk and in a highly purified condition, was emulsified in water and inoculated with the experimental cultures, growth was observed in every case. In this preparation some of the cultures developed only in the depth of the liquid, but the species that produce the most extensive growth on butter developed reasonably well on the surface. When a solid substrate was afforded the cultures, the growth was much more marked.

Everyone familiar with the butter industry appreciates that molding most often occurs after butter has been stored for a time. During this time the fats may be hydrolyzed so that glycerol and the constituent acids are released. In this connection, it was desirable to

consider the value of glycerol and some of the organic acids commonly present in the mixed glycerides known as fat. It was found that glycerol was not a particularly favorable source of food for the cultures used, at least in the concentration employed. Development below the surface, in almost every instance, was much better than that on the surface of the medium, whether the glycerol was in a liquid or a solid medium. Butyric acid was the only water-soluble fatty acid studied. In the one per cent aqueous solution of this common hydrolytic product of butterfat, growth was not observed in any instance. Apparently it was toxic for the cultures studied. The growth in the unsaturated oleic acid was much better than that obtained on the saturated palmitic acid, which in turn appeared to be more nearly suited to the demands of the molds studied than the other typical saturated fatty acid, stearic. As oleic acid predominates in butterfat, on some occasions it may be called upon to furnish the food supply of molds.

In a general way butterfat, lecithin, and related substances, were not particularly satisfactory sources of food for the early development of the conidia of the mold cultures used. Normal growth was not obtained in any of these substances, even where sufficient quantities of water were present. It is conceivable, however, that these compounds may be utilized readily by molds that have made their early development of mycelium upon substrata furnishing more suitable food supplies, and after the enzymes necessary to split the fats into readily assimilable compounds have been formed. Many of the typical flavors and odors produced in butter and cheese, such as those characteristic of Roquefort cheese, are associated with fat hydrolysis by molds. Such a flavor and aroma was noticeable in some of the fat, water, and agar combinations before there were any visible signs of mycelium. This hydrolysis of the fat may have been the result of enzymes carried by the inoculum, but it is possible that the germinated hyphae produced enzymes when they were called upon to supply nourishment, with fat as the only available source of food. Fat and its related compounds must be considered as potential sources of food for mold growth.

Nitrogen is one of the most important of the elements for the metabolism of the living cell. Many nitrogen-bearing compounds are suitable for this purpose but natural proteins, their split products (peptone, amino acids, etc.), and related compounds are generally considered the most satisfactory sources of nitrogen for microörganisms. Organisms that can obtain their nitrogen directly from the atmosphere as elementary nitrogen, are known but the species under investigation have not been shown capable of fixing nitrogen in this manner. Rather, they must use other sources of supply. The part of butter that fur-

nishes nitrogen compounds in considerable amounts is known as the curd. This is really a misnomer, as casein, albumin, and similar nitrogenous complexes normally found in milk are carried over into the butter in much the same form as they exist in milk, or more correctly, in buttermilk. This buttermilk is held in butter principally in the form of tiny droplets, with a full quota of the water, which makes up the bulk of buttermilk. As a starting point in the investigation of the value of nitrogenous substances as a source of food for molds, solutions of peptone were used. The germination and hyphal development of the cultures on this substrate were both rapid and luxuriant, and growth was as extensive as might be desired. In contrast with the tendency toward subsurface development in the fatty substances, the growth on peptone was predominately on the surface. The more noticeable surface development of the cultures upon lecithin preparations than upon fats or fatty acids may be due to a nitrogen-bearing radical in the lecithin molecule.

In order to obtain an idea of the usefulness of the actual curd portion of butter for the growth of molds, the fat was removed from a sample of typical butter so that the curd and water portion became available. (See page 35.) This substance consisted of a mixture of casein, albumin, lactose, and mineral salts, with about 85 per cent of water. The growth of the test cultures on this substrate was prompt and luxuriant. When this material was dialyzed to remove some of the soluble constituents, such as lactose, mineral salts, and other diffusible compounds, the residue served adequately as a source of food for the molds being studied. While growth was not quite so rapid as upon the original mixture, it became equally extensive in a short time. This demonstrated that the important constituents were not diffused through a collodion membrane and indicated that the principal sources of subsistence were the colloidal compounds of nitrogen.

As a further test of the non-fatty portions of butter, which were to be considered as largely nitrogenous, from the theoretical standpoint at least, the sera obtained from the successive washings and separations of cream (see page 36) were employed as substrata for the cultures. The first washings were found to be satisfactory sources of nutrients but each successive washing left less and less material for the needs of a growing cell. This may be considered to indicate that a rich source of food is incorporated in butter in the form of the solids-not-fat in cream and that the purification of fat by successive washings and separations may remove so much of the food for fungi that growth would be materially retarded in the finished product consisting largely of fat. Preliminary studies now under way lead the writer to believe that this is actually the case.

As lactose is a constituent of butter, an investigation was undertaken to determine its suitability as a food for the experimental cultures. In an aqueous solution of lactose, growth was very meager, especially on the surface. Some subsurface mycelial development was noted, especially in the bottom of the liquid. When the lactose was held in a washed agar medium, growth was somewhat better, especially in the depth of the agar. A penetration of the substrate occurred with all cultures. The species of Hormodendrum and Alternaria grew luxuriantly below the surface and carried a deep green and almost black color in their mycelia; very scanty growth appeared on the surface.

Lactic acid, which is so commonly formed by the fermentation of lactose, did not prove a satisfactory food source and, in general, growth of the molds studied was poor at the concentration used.

The diffusate obtained by the dialysis of the curd of butter was a much more satisfactory substrate than pure lactose solutions. This may be attributed to the presence of soluble nitrogen compounds and mineral salts in addition to lactose. At any rate, with the diffusate the growth was limited almost entirely to the surface of the liquid, which is suggestive of the results obtained with nitrogenous substances. Development was most active within a few days, and continued incubation did not significantly increase the visible mycelium. This indicates an early exhaustion of essential food elements. The belief that nitrogen compounds are the principal sources of nourishment was substantiated in part by an observation on the pH of the substrate in different cultures. The initial pH of the diffusate was 6.4. After 3 weeks the hydrogen-ion concentration of the medium seeded with Cultures 6 and 7, which developed the least, was pH 6.2 in each case. In the other cultures, the reaction was altered to points from pH 7.0 to 7.6, which was suggestive of alkaline by-products of protein hydrolysis.

Lactose was not satisfactory material for the growth of the test cultures. The more abundant growth in the depth of the medium was in accordance with a similar phenomenon in the case of substrata containing glycerol, fatty acids, and fats, all of which are compounds lacking nitrogen. The fact that subsurface growth was also noticeable in the non-nutritive washed agar medium, which served as a check, might indicate that where the food supply was not especially favorable the tendency was toward deeper penetration of the substrate. This is not an entirely satisfactory explanation, however. The species of Hormodendrum and Alternaria showed a tendency to spread into the depth of butter, much as they did on the above-mentioned media, and produced similar dark-colored mycelium even when an adequate and desirable food supply was available. Some reason other than the lack of suitable food must be sought to explain this situation.

Almost everyone will concede that mineral elements of various sorts are essential for the growth of microörganisms. Fortunately, minute traces are ordinarily sufficient to meet the needs of the living cell. In a natural product like milk, they usually exist in combination with other constituents or as impurities, even after attempted purifications. Molds would not be expected to grow in such a highly alkaline preparation as a solution of milk ash. The results presented demonstrate this. However, a 0.75 per cent solution was not sufficient to destroy the conidia, as evidenced by the fact that they were capable of normal growth when sources of nitrogen, carbon, etc., were supplied. It is surprising that growth occurred in such an ash solution even when it was neutralized, as theoretically no nitrogen or carbon was present. These elements might have been supplied from some other source when molds were able to grow in a neutralized ash solution.

Growth of the cultures in mixtures of butterfat, lecithin, and water was not conspicuous. The surface tension may be a factor in this, as both of the former substances depress the surface tension, the combination perhaps more than either alone. The presence of particles of milk ash on butterfat, in an environment well supplied with water, appeared to encourage the development of certain species but could not be considered as giving good growth. When the solutions of ash were mixed more intimately with the fat, the growth was much better. This probably means that the mineral salts added a stimulus for the attack upon fat.

The results with a combination of peptone and lactose in liquid or solid media indicated how important is a nitrogenous compound for the development of fungi. While the proportion of growth on the surface of the medium was much greater than on simple lactose substrata, there was a greater tendency for subsurface mycelium to develop than in pure peptone solutions. This may mean that there is something fundamental about the mode of development of molds upon nitrogenous and non-nitrogenous media. The disturbance in the normal surface development of molds in the presence of such substances as lactose, glycerol, fatty acids, and fat is striking.

Altogether, butter provides a wide variety of foodstuffs, quite sufficient to sustain the growth of molds. The important consideration is not the amount or quality of the food, however, but its availability. The physical structure of butter must be taken into account, as the most desirable nutrients are dispersed in minute droplets throughout the mass of fat. If the molds happen to be situated where this food is accessible, and if other factors are favorable, growth should take place freely.

Moisture.—The effect of moisture on the growth of molds is clear. All living protoplasm has a relatively high moisture content and

the normal nutrition of the cell takes place from a food supply dispersed in an aqueous dispersion medium.

Moisture-free fat is not a good substrate for growth. Butter regularly carries a reasonable amount of moisture even upon a basis of percentage total composition. When it is borne in mind that butter is more than 80 per cent fat, and that the water, constituting from 12 to 16 per cent of the total weight, is largely in association with the nitrogenous compounds as well as the lactose, mineral salts, and various traces of other compounds, in the form of buttermilk droplets, it may be appreciated that there should be a plentiful supply of moisture for the growth of molds. It has already been pointed out that by actual analysis the curd portion of butter was about 85 per cent water. Therefore it may be assumed with reasonable assurance of correctness that the moisture conditions in normal butter are perfectly satisfactory.

The humidity of the atmosphere, however, is not such a constant factor, especially in relation to the outer surface of butter. Sometimes butter is stored in a dry, well-ventilated refrigerator, or again in a damp place where the air is stagnant and evaporation slight so that the moisture on the surface of the butter is retained. The results of the experiments reported in preceding pages indicate that humidity may play a considerable part in the development of molds on butter. There is a significant fact which stands out in this connection, however. It appeared that sterile butter, inoculated on the surface with mold spores, failed to show any mold growth when kept in a storage room at a low humidity but extensive growth took place when the atmosphere was saturated with moisture. On the other hand, butter made from cream in which molds had been growing for several days developed moldy areas even under conditions of low humidity. Apparently a fundamental difference is involved. In the first lot of butter the conidia found it difficult or impossible to germinate because of the lack of moisture brought about by evaporation on the surface; in butter in which molds were growing actively, the process was checked only temporarily during churning and continued afterwards because of the abundant supply of moisture obtained through the mycelium from the reservoirs of water in the droplets of buttermilk. This feature may be of practical significance. If infection takes place before churning and the molds are able to establish a mycelial development before the butter is made, growth may continue and the surface of the butter be marred in appearance, even tho the product be stored in an atmosphere low in moisture and conducive to rapid evaporation. On the other hand, if contamination takes place after the butter is made, and the finished product is kept at a low humidity, mold may not appear. Undoubtedly, a dry, well-ventilated storage room may be an important factor in preventing the molding of butter.

Temperature.—The temperature at which mold growth occurs varies decidedly with the species of molds and the nature of the substrate. The results of the experiments reported demonstrate clearly that *Aspergillus niger*, and *Aspergillus flavus* were checked when the temperature of incubation was below 10° C. This indicates that these species need not be considered as responsible for the discoloration of butter as long as the product is kept at low temperatures. The growth of *Rhizopus nigricans* was inhibited at a temperature of 0° C. Species of Penicillium, Alternaria, and Hormodendrum were not so sensitive to low temperatures. This is significant, as these species are among the most common causes of molding in butter, even when it is kept at low temperature. The amount of salt in the butter also affected the growth of the various species at different temperatures. It had a more noticeable effect on the growth of the molds at low than at high temperatures. Time is a very important factor in the growth at lower temperatures. As might be expected, species which are able to grow at low temperatures may require considerable time before they can produce noticeable growth, also the species that are least inhibited by salt grow at the lower temperatures. As the amount of salt in the butter has a marked effect upon the freezing point of the water droplets containing the food materials, with a high salt content the nutrients may remain in a liquid substrate at temperatures much below the ordinary freezing point of water. Consequently, species able to grow at low temperatures and at high salt concentrations may bring about the molding of butter in storage if time is allowed for their growth. The temperatures used in these studies were not especially low, so it would be desirable to continue the investigations at much lower temperatures with species known to be common causes of molding in butter.

Atmosphere.—The effect of atmospheric conditions, aside from humidity, upon the growth of molds, is worth consideration. The results of the experiments reported here indicate that a sufficient supply of oxygen is essential for the development of the ordinary molds. When the amount of available oxygen was reduced by a partial evacuation of the air within a sealed container in which infected butter was stored, the growth was retarded but, in time, development began. It is possible that oxygen was released from the food constituents after the atmospheric oxygen was consumed and eventually permitted satisfactory growth of most of the species studied. Where the oxygen was removed so that anaerobic conditions existed, no growth of molds occurred. Butter is seldom stored where oxygen is completely excluded. Sometimes it is placed in containers upon which a partial vacuum is drawn. Consequently, under ordinary commercial conditions, the supply of oxygen may be considered reasonably satisfactory.

In sealed containers, mold growth may appear, as it sometimes does in canned condensed milk. This condition was studied in the experiment in which butter was kept in a partial vacuum. It is not economically feasible, however, to handle butter where all oxygen is removed. It may be possible to replace the oxygen by some inert gas, but experiments are necessary to demonstrate the practicability of such a plan. The partial removal of carbon dioxide from the atmosphere in which butter was stored did not retard mold development in the experimental samples. How essential carbon dioxide is for mold growth has not been determined, but further studies should be made to ascertain this point. Without doubt, atmospheric conditions influence mold development, but to what extent is not yet evident. The fact that certain species were able to grow so extensively in the depth of media containing carbohydrates, fatty acids, glycerol, etc., raises a question as to these relationships.

Salt.—The amount of salt in butter is unquestionably an important factor influencing the growth of molds. The results presented in the foregoing pages demonstrate that the effect of salt upon growth depends especially upon the species of molds, the amount of moisture, and the temperature. In accordance with many previous investigations, *Oospora lactis* was checked by a slight concentration of sodium chloride in the substrate. The disappearance of this fungus in salted butter may be explained on this basis. The species of Mucor and Rhizopus studied were only slightly more resistant to salt and consequently would not be expected to be important in the molding of salted butter. *Aspergillus niger* and *Aspergillus flavus* are resistant to high salt concentrations, but when butter was kept at temperatures below 10° C. growth was impeded. For this reason, their importance as causes of molding in butter stored at the usual commercial temperatures is slight. The other molds studied—Alternaria, Hormodendrum, and Penicillium—were found to be capable of growing in high salt concentrations and at the lower temperatures. As these species produce the most marked changes in the appearance, flavor, and aroma of butter when they are able to develop, they are of major importance. The species of Alternaria and Hormodendrum studied produced dark green, almost black, smudgy areas. Their mycelia spread considerable distances from the point of infection, both along the surface and to considerable depths in the butter. The species of Penicillium used did not mar the appearance of the butter particularly, altho in some cases sufficient green fruiting bodies were formed to give a slight, dusty discoloration of the surface. The cultures of Penicillium affected largely the flavor and aroma. Butters in which these species were growing became distinctly cheesy in flavor and odor, and in these characters suggested Roquefort cheese. In any consideration of the effect of salt on the growth of mold

in butter, the concentration of the salt in the droplets of water within the butter must be taken into account. Even tho a gravimetric analysis of butter may indicate a high salt content, it is important to know how much water is present to carry this salt. Some of the experiments reported in the preceding pages illustrate this point. If the percentages of water and salt are both high, the concentration of salt in the water may not be any higher than if both are low. Equally, if the water content is low and the salt content moderately high, the brine may be highly concentrated. Then, also, the salt may be unevenly distributed in the minute droplets of moisture within the butter. Some may represent a strong and others a weak brine. The number of conidia in butter, even under extreme conditions, will seldom be large and never considerable in proportion to the number of water droplets. It is conceivable that a conidium may be allocated to a droplet of water containing little or no salt and an abundant supply of food and accordingly be able to germinate and develop without restraint. Only one such instance would lead to serious consequences as far as molding is concerned. Thus the effect of salt on the growth of mold on butter will depend entirely upon the relative concentration of the salt in the droplets in which the mold spores, or mycelium, may be located. This may partly explain the molding of salted butter, even tho the composition of the butter and type of mold may at first have been considered sufficient reasons for expecting protection against such a contingency.

Species of molds.—It is clearly evident that the species of molds must be considered as factors in the molding of butter. A wide variety of species has been isolated from butter. Among these, the ten selected for the studies reported were all capable of growing in butter under favorable conditions. From the standpoint of visible growth, *Oospora lactis* was least noticeable. It seldom appeared on the surface of the inoculated butter but evidently was able to develop in unsalted butter, as shown by the fact that in nearly every instance the odor of the butter became distinctly cheesy, resembling most closely the odor of Cheddar or Brick cheese. *Aspergillus flavus* produced a slight, white felt upon the butter and also fruited extensively to produce yellow or yellowish-green sporangia above the surface of the butter. It produced a rather indefinite change in the aroma of the butter that was suggestive of fat hydrolysis and bordered on that produced in Roquefort cheese. *Aspergillus niger* likewise formed a white, cottony layer of mycelium from which a large number of chocolate-brown or black sporangia arose. The odor produced in the butter was much the same as that observed with *Aspergillus flavus*. *Alternaria humicola* grew extensively in the form of a white, surface mycelium that eventually became dark green and penetrated into the butter to cause a very dark green or black smudge. The odor produced was peculiar, with a suggestion of acetic

and butyric acids as well as a slight cheesiness, resembling a rather poor quality of Cheddar. *Hormodendrum cladosporioides* grew rapidly and extensively, producing dark green fruiting bodies promptly and sending dark green or black hyphae into the butter and spreading widely over the surface. The principal change in the odor of the butter was the development of mustiness, with a faint suggestion of the aroma of an old cottage cheese. *Mucor sylvaticus* sent up an extensive aerial mycelium terminating in numerous grayish or black sporangia. The odor of the butter was rather indefinite but resembled acetic and butyric acids, and also suggested tallow. *Penicillium expansum* and *Penicillium biforme* produced white, cushion-like spots which, in time, became gray-green to blue-green. The aroma of both species was decidedly like that of Roquefort cheese, altho some other indefinable odors were noticeable. The observation that the Penicillium species were capable of producing the characteristic odor of Roquefort cheese in butterfat and butter when there were no visible signs of growth deserves mention. In a similar way, other molds may bring about alterations in the flavor or aroma of butter without affecting the appearance. *Rhizopus nigricans* grew well on the unsalted butter and appeared as a mass of aerial mycelium topped with many black sporangia. The odor was not markedly affected altho a slight acetic acid aroma appeared in time. As may be seen, most of the mold species studied grew well in butter when humidity, temperature, atmosphere, and salt concentration were favorable, and produced evident changes in the appearance and odor of the butter. The other species of molds isolated should be studied in a similar manner, to determine which can actually grow in butter and, if growth is possible, what conditions influence the development.

It is evident that, qualitatively and quantitatively, butter contains a satisfactory supply of food for mold growth. Whether or not molds will develop upon a given lot of butter depends upon several factors, among which must be considered, especially, the extent of contamination, the species of molds, the supply of oxygen, the temperature, and the concentration of salt in the aqueous portion of the butter carrying the major portion of the most useful food constituents. A great deal of work remains to be done to elucidate some of these points.

SUMMARY

1. Studies were made of some of the factors influencing the growth of molds in butter.

2. The ten species of molds isolated from butter and selected for the investigations, were as follows: *Alternaria humicola*, *Aspergillus flavus*, *Aspergillus niger*, *Hormodendrum cladosporioides*, *Mucor syl-*

vaticus, Oospora lactis var. A., Oospora lactis var. B., Penicillium biforme, Penicillium expansum, and *Rhizopus nigricans.*

3. The influence of the food supply, moisture, atmosphere, temperature, and salt concentration upon the growth of the ten species was studied.

4. Purified butterfat was not a readily utilizable food, unless water was present. In that case, growth was only moderate, however.

5. Lecithin proved to be a reasonably satisfactory food for the molds studied.

6. The hydrolytic products of fat that provided fairly satisfactory nutriment were glycerol, palmitic acid, and oleic acid. *Alternaria humicola* made slight growth on media containing stearic acid as the only source of food. No growth occurred on a one per cent solution of butyric acid.

7. Compounds containing nitrogen, such as peptone, curd from butter, and serum from cream, were found to be excellent sources of food. Growth was luxuriant on the substrata containing these nitrogenous substances.

8. Lactose and lactic acid in one per cent solutions did not furnish especially good nutriment.

9. Solutions of milk ash were not readily utilized by the molds studied unless they were neutralized.

10. Combinations of fat, lecithin, and water; fat and ash; fat, ash, and water; and lactose and peptone, provided far better growth than was obtained on the single substances.

11. The molds grew most extensively on the surfaces of media containing nitrogen-bearing compounds.

12. The growth on substrata containing fats, fatty acids, glycerol, lactose, or lactic acid was largely below the surface of the medium.

13. Unsalted butter containing a mixture of fat, protein, carbohydrate, and ash supported active growth.

14. The humidity of the atmosphere had a marked influence upon the growth of the molds on butter, especially when the surface of the butter was contaminated. At low humidities growth was checked. When the molds were actively growing in the cream before the butter was made, the humidity had a less pronounced effect upon the growth.

15. Temperature exerted a marked influence upon the growth of the species in various substrata. Growth was active in all at 20° to 25° C. *Aspergillus flavus* was checked at a temperature of 10° C. or lower; *Aspergillus niger* and *Rhizopus nigricans* at 0° C. *Mucor sylvaticus* grew in whey media and buttermilk, but not in butter kept at 0°. The other species were able to develop at 0° C. but the growth was not so extensive or so rapid as that at 10° and 20° C. Time is a factor influencing the growth at low temperatures.

16. The species of molds studied were able to grow on butter at 20° to 25° C. when under a vacuum of 25 inches.

17. Partially removing carbon dioxide from the atmosphere did not prevent the growth of the molds on butter.

18. None of the molds were able to develop when the oxygen was exhausted from the atmosphere.

19. Salt exerted a marked effect upon the growth of certain species.

20. *Oospora lactis, Mucor sylvaticus,* and *Rhizopus nigricans* were most readily affected and their growth was inhibited when the concentration of salt exceeded 5 per cent.

21. *Alternaria humicola, Aspergillus flavus, Aspergillus niger, Hormodendrum cladosporioides, Penicillium biforme,* and *Penicillium expansum* were capable of growing in media containing 15 per cent of salt. In some cases, the species of Penicillium showed slight growth when the percentage was as high as 20 per cent.

22. The extent to which salt inhibited the growth of the molds studied depended upon the species of molds, and the temperature of incubation.

23. All the species of molds studied were able to grow on butter when conditions were favorable.

24. The appearance of the butter was marred most extensively by *Alternaria humicola* and *Hormodendrum cladosporioides.* The flavor and odor of the butter were affected seriously by all the other species.

25. As an ample food and water supply are provided, the growth of molds in butter appears to depend largely upon the species of mold, the humidity of the atmosphere, the supply of oxygen, the temperature of storage, time, and the concentration of salt. These influences may act separately or collectively.

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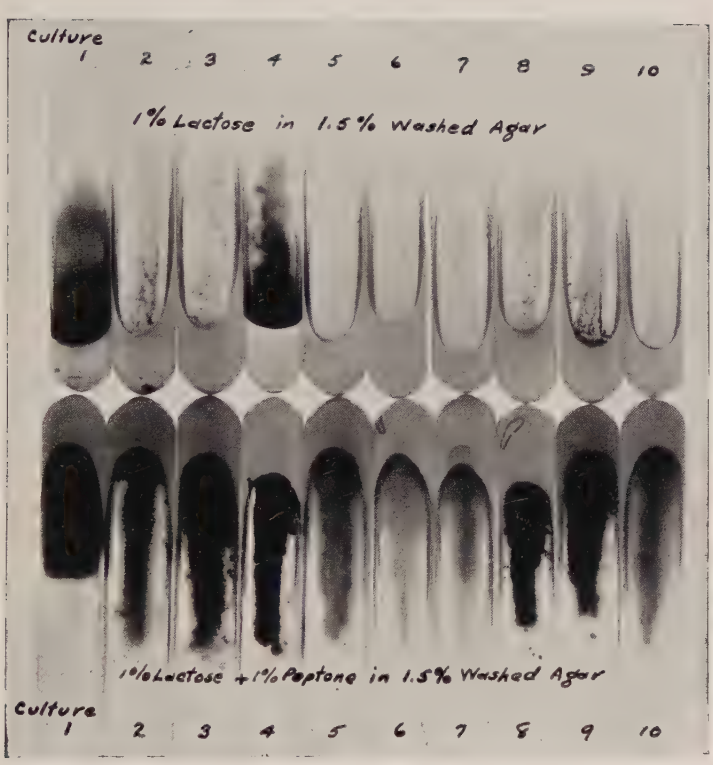

Plate 1. Comparative Growth of Test Cultures on One Per Cent Lactose in 1.5 Per Cent Washed Agar and on One Per Cent Lactose + One Per Cent Peptone in 1.5 Per Cent Washed Agar

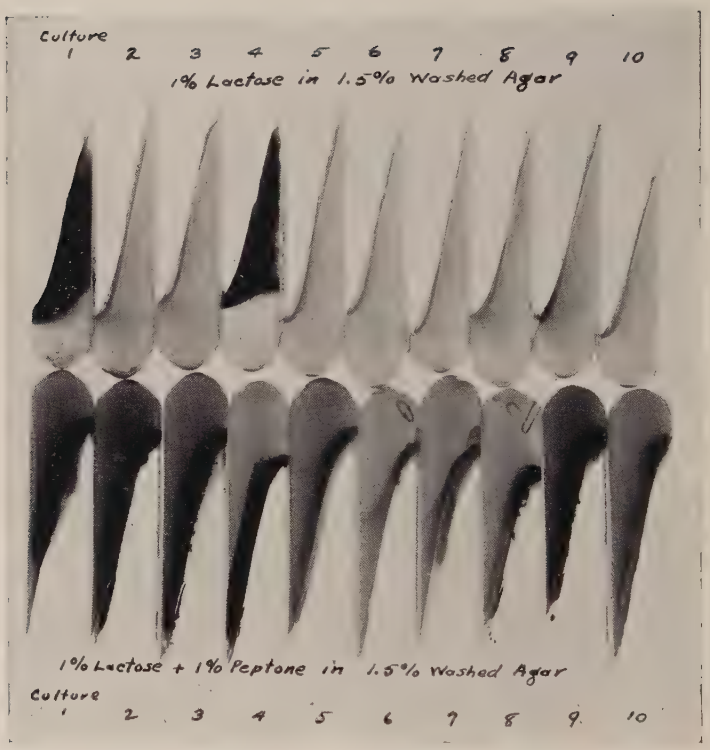

Plate 2. Penetration of Mycelium into Substrate

The apparent clouding in the medium containing lactose and peptone together is due largely to shadows cast from the surface mycelium.

Plate 3. Typical Appearance of Growth of Test Cultures on Unsalted Butter

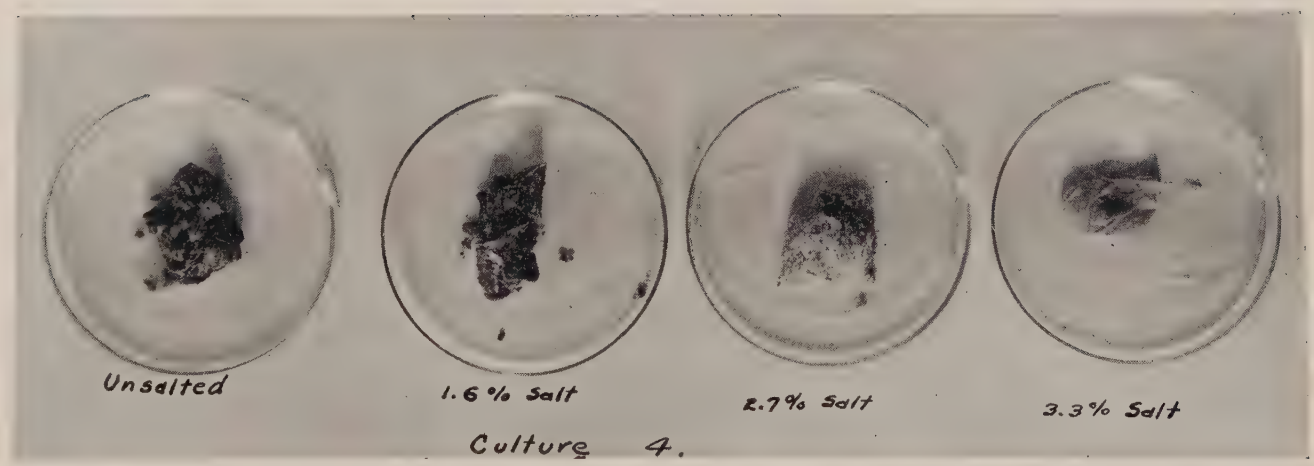

Plate 4. Effect of Salt Concentration upon the Growth of *Hormodendrum cladosporioides* in Butter